

IRON REDUCTION MEDIATED INCREASES IN CARBON OXIDATION AND  
PHOSPHORUS PRECIPITATION IN ON-SITE WASTEWATER SYSTEMS

BY

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DISSERTATION

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## ABSTRACT

1.2 billion people lack access to safe drinking water, 2.6 billion have little or no sanitation, and millions of people die annually from diseases transmitted through unsafe water and human excreta. Onsite wastewater systems (OWSs), referred to as septic tanks, serve approximately 25% of the United States population. Septic systems typically operate under strictly anoxic conditions, and fermentation is the dominant process driving carbon transformation. Moreover, phosphorus, a major nutrient that causes eutrophication, accumulates in onsite wastewater systems from human and domestic wastes. Phosphate is of particular interest because of its growth limiting effects on microbes in natural aqueous environments. We proposed a novel technique to anaerobic onsite wastewater by leveraging iron reduction to oxidize more carbon and precipitate phosphorus with reduced iron as vivianite.

We used carbon-14 radiolabeled acetate, lactate, propionate, butyrate, glucose, starch, xylose and oleic acid to demonstrate that short and long term carbon oxidation is increased when different forms of  $\text{Fe}^{3+}$  are amended to septic wastewater. The rates of carbon mineralization to  $^{14}\text{CO}_2$  increased 2 to 5 times (relative to un-amended systems) in the presence of  $\text{Fe}^{3+}$ . The extent of mineralization reached 90% for some carbon compounds when  $\text{Fe}^{3+}$  was present, compared to levels of 50-60% in the absence of  $\text{Fe}^{3+}$ . When methanogenesis was the dominant process present in-situ;  $^{14}\text{CH}_4$  was not generated due to  $\text{Fe}^{3+}$  amendment, demonstrating that this strategy can limit methane emissions from septic systems. Moreover, iron reduction as a terminal electron accepting process increases the microbial diversity of the wastewater. Amplified 16S rDNA restriction analysis (ARDRA) confirmed that unique  $\text{Fe}^{3+}$ -reducing microbial communities increased significantly in iron-amended incubations.

Pure culture growth experiments in ferric citrate media with *Geobacter metallireducens* clearly showed extensive iron reduction and subsequent phosphate (>97.5% or higher) removal

in presence of electron donor. Both TEM-EDAX and XRD techniques confirm the precipitant as vivianite when additional  $\text{Fe}^{2+}$  was present. Ferric citrate, ferrihydrite and ferrihydrite+AQDS completely removed (>95.5% or higher) of phosphate in the original wastewater sample. Ferric citrate and ferrihydrite+AQDS performed better (>97.5 or higher) when an extreme phosphate loading condition was evaluated.

The form of  $\text{Fe}^{3+}$  added had a significant impact on the rate and extent of mineralization with ferrihydrite and lepidocrocite favored as solid phase  $\text{Fe}^{3+}$  and chelated  $\text{Fe}^{3+}$  (with NTA or EDTA) as preferred soluble  $\text{Fe}^{3+}$  forms. Though chelated  $\text{Fe}^{3+}$  (with NTA or EDTA) removed phosphate at a significant level; ferric citrate, ferrihydrite and lepidocrocite showed quick and high phosphate removal from wastewater sample. The result clearly shows that iron reduction coupled with phosphate removal can be an effective strategy for onsite wastewater systems.

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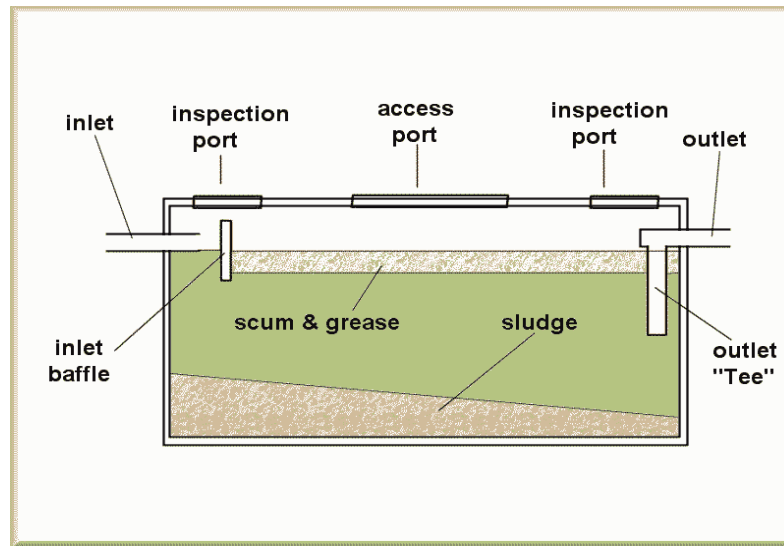
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## CHAPTER 1 INTRODUCTION

1.2 billion people lack access to safe drinking water, 2.6 billion have little or no sanitation, and millions of people die annually from diseases transmitted through unsafe water and human excreta (Shannon et. al., 2008). Onsite wastewater treatment systems (OWS), also referred to as septic systems are dominant in developing countries. Moreover, 25% of the US population also depends on decentralized wastewater treatment (Conn et al, 2006, Lowe and Siegrist, 2008). This percentage amounts to OWSs serving 22 million businesses and homes throughout the United States (Tomaras et al., 2009). OWSs treat and distribute wastewater from individual homes, small communities, and commercial buildings (Tomaras et al., 2007).

Generally associated with “septic tanks,” on-site wastewater systems transform and treat human and household waste through complex physical, chemical, and biological processes (McCray and Christopherson, 2008). Advanced onsite wastewater treatment typically involves anaerobic septic tanks, followed by additional treatment units such as aerobic bioreactors, sand filters or constructed wetlands. Prefabricated septic tanks serve as a combined settling and skimming tank, as an unmixed-unheated anaerobic digester and as a sludge storage tank (Crites and Tchobanoglous, 1998). Settleable solids form a sludge layer at the bottom of the tank and flocculent materials such as oil and grease form a scum layer at the top of the aqueous portion of the tank. The detailed configuration of typical septic tank is shown in Fig. 01.



**Figure 1:** Typical configuration of septic tank.

(Source: <http://www.title5v.com>)

While stable OWS are effective for domestic wastewater, they are relatively inefficient; converting only 30-50% of  $BOD_5$  (Lowe et al., 2007) to mineralized end products, such as  $CO_2$  or  $CH_4$  (Crites and Tchobanoglous, 1998) within the tank. In addition, many systems generate more  $CH_4$  than  $CO_2$ , which is a concern because methane is a more potent greenhouse gas than carbon dioxide (US EPA, 2009). The carbon that does not get oxidized to either  $CH_4$  or  $CO_2$  ends up in: i) the sludge layer, ii) the scum layer, or iii) the aqueous effluent that enters the natural environment (Crites and Tchobanoglous, 1998).

Moreover, domestic wastewater is one of the major sources of phosphates and nitrogen contamination (Svanks, 1971). Human waste usually contains orthophosphate and organic phosphorus such as nucleic acids, phospholipids and phosphorylated proteins. In domestic and industrial wastes, phosphorus may also be present as condensed phosphates (pyrophosphate or tripolyphosphate) that often originate from detergents (De Jong, 1985). The need to control phosphorus loading to receiving waters and prevent eutrophication has been well documented (Vollenweider, 1985). Eutrophication has many negative effects on aquatic ecosystems such as

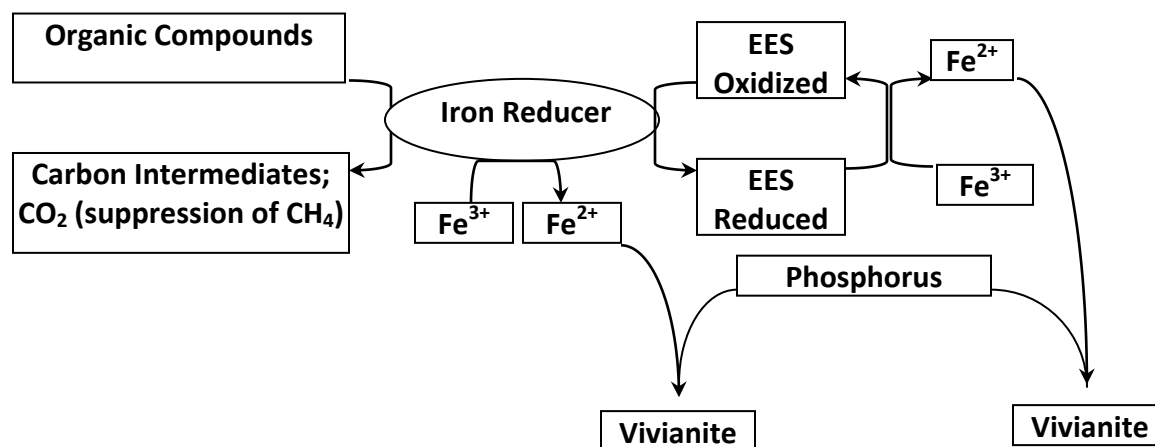
proliferation of algae, degradation of water quality, oxygen shortages, loss of natural habitats, and negative impacts on aquaculture and shellfisheries etc (Carpenter et al., 1998). Coastal and estuarine environments are particularly sensitive to nutrient inputs (Lapointe et al., 2005). As a consequence, phosphorus is of greatest concern when septic systems are located close to sensitive surface water bodies.

Septic systems typically operate under strictly anoxic conditions, and fermentation is the dominant process driving carbon transformation. Carbon oxidation is limited when compared to municipal wastewater treatment, because fermentation does not transform the same number of carbon molecules as aerobic respiration in absence of external electron acceptor. Nitrate,  $\text{Fe}^{3+}$ , and sulfate reduction may be operating to a limited extent in any given septic tank, but the total carbon oxidation that can be attributed to these processes under native conditions is minimal.

$\text{Fe}^{3+}$  reduction is not a dominant reaction pathway in septic systems. Typically fermentation, methanogenesis, and perhaps sulfate reduction are the most prevalent reactions (Crites and Tchobanoglous, 1998). However,  $\text{Fe}^{3+}$  reducers are present and inactive (or with limited activity), and easily stimulated by adding  $\text{Fe}^{3+}$ .  $\text{Fe}^{3+}$  reducers can metabolize a broader array of carbon compounds than sulfate reducers, methanogens, or fermenters.  $\text{Fe}^{3+}$  reducers oxidize carbohydrates, lipids, and organic acids to  $\text{CO}_2$ , without accumulating intermediate carbon compounds (Coates et al., 1996a; Coates et al., 1998).

Therefore, adding  $\text{Fe}^{3+}$  to septic systems can increase the rate and extent of total carbon oxidation by allowing  $\text{Fe}^{3+}$  reducers to proliferate and become the dominant microbial community. Different types of iron can be utilized by a wide variety of iron reducing microorganisms in natural and laboratory environments (Fredrickson and Gorby, 1996; Liu et al, 2002; Nealson and Saffarini, 1994; Lovley, 2000). Anthraquinone-2,6-disulfonate (AQDS)

(known as electron shuttling compounds-EES) has been used to increase the reductive dissolution of synthetic or natural, amorphous (Fredrickson et al., 1998) or crystalline  $\text{Fe}^{3+}$  (hydr)oxides in laboratory studies (goethite, hematite; Zachara et al., 1998). Iron reducers can degrade complex molecules like lipids or pharmaceuticals. Moreover,  $\text{Fe}^{2+}$  produced from  $\text{Fe}^{3+}$  reduction can combine with phosphorus to precipitate vivianite in septic wastewater. This can reduce the risk of phosphorus disposal and subsequent eutrophication impacts significantly. Thus, a novel integrated technique to septic wastewater is important to reduce environmental risk of carbon disposal, methane emission and phosphorus removal. The conceptual model of the research is shown below:



**Figure 2:** Graphical representation of overall conceptual model of research.



## CHAPTER 2 LITERATURE REVIEW

### 2.1 Introduction

Phosphorus is a nutrient that accumulates in wastewater from human, domestic and industrial wastes (Alexander & Stevens, 1976; Barnes et al., 1984) and run-off from phosphorus-rich fertilized land (Kauppi, 1985). Phosphate is of particular interest because its growth limiting effects on microbes in natural aqueous environments (Schindler, 1977; Hudson et al., 2000; Wu et al., 2000). Domestic wastewater is one of the major sources of phosphates and nitrogen contaminants (Svanks, 1971). The need to control phosphorus loading to receiving waters and prevent eutrophication has been well documented (Vollenweider, 1985). Coastal and estuarine environments are particularly sensitive to nutrient inputs (Lapointe et al., 2005). As a consequence, phosphorus is of greatest concern when septic systems are located close to sensitive surface water bodies.

Human waste usually contains orthophosphate and organic phosphorus such as nucleic acids, phospholipids and phosphorylated proteins. In domestic and industrial wastes, phosphorus may also be present as condensed phosphates (pyrophosphate or tripolyphosphate) that often originate from detergents (De Jong, 1985).

A typical anaerobic sludge (such as septic tanks) is a complex mixture of minerals and organic matter (Fletcher and Beckett, 1987). Phosphorus often combines with a number of metal cations such as iron, aluminum, manganese, and calcium, to form a wide range of minerals that can be stable in low-temperature aqueous environments (Nriagu and Dell 1974; Stumm and Morgan 1981). Most soluble inorganics in an anaerobic digester are complexed with ligands. The inorganics are balanced between: solid precipitates, co-precipitates/ions sorbed to inorganic

precipitates, ions adsorbed to and complexed with organic material; ions adsorbed to and accumulated within microorganisms (Gould and Genetelli, 1975).

$\text{Fe}^{3+}$  (solid or soluble) reduction is a form of respiration that is coupled to energy production (ATP) and cell division (Lovley, 2000). The resultant  $\text{Fe}^{2+}$  can end up in many forms (depending on pH,  $E_h$ ,  $\text{Fe}^{3+}$  form, and prevailing geochemistry).  $\text{Fe}^{3+}$  reducing microorganisms are ubiquitous, including in septic sludge; however, in these systems they are limited by low (or absent) bioavailable  $\text{Fe}^{3+}$  (Castillo-Gonzalez and Bruns, 2005; Coates et al, 2005; Ivanov et al., 2004; Kim et al, 2004).

Phosphorus mineral precipitation is practically acceptable because it immobilizes phosphorus as solid compounds without the need for excessive biomass or additional oxygenation. Thus, overall sludge load of the system can be reduced, which is critical to efficient sludge treatment (Wang et al, 2005) and unit operations. In addition, these minerals are more stable than biologically accumulated sludge (Wang et al, 2005). To date chemical precipitation has been suggested or reported (Santruckova et al, 2004; Sondergaard et al, 2003; Wang et al, 2005) but biological precipitation is possible with no additional infrastructure requirement. The research work has been focused on biological phosphorus removal via secondary reactions catalyzed by  $\text{Fe}^{3+}$  reduction, namely vivianite production ( $\text{Fe}_3(\text{PO}_4)_2 \cdot (\text{H}_2\text{O})_8$ ).

## 2.2 Phosphorus Removal

Phosphorus removal from wastewater can be achieved either through chemical removal, advanced biological treatment or a combination of both (Wang, *et al.*, 2006). The chemical phosphorus removal techniques involve the addition of calcium, iron and aluminium salts to achieve phosphorus precipitation by various mechanisms (Yeoman et al, 1988). Generally, aluminium sulphate (alum) is considered the best precipitant (Lin and Carlson, 1975), followed

by  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{Fe}_2(\text{SO}_4)_3$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{Ca}(\text{OH})_2$  (Metcalf and Eddy, 1993). Environmental factors such as pH, Eh, alkalinity, coagulant dose, speed of mixing, microbial activity and other interfering substances have significant effect on chemical phosphorus removal (James, *et al.*, 2003).

### ***Mechanisms of phosphorus removal***

#### **2.2.1 Calcium**

Lime is the most common calcium salt used for phosphorus removal from wastewater (quicklime or almost pure calcium oxide). The reaction forms hydroxyapatite within the pH range of 8 to 11 according to the following reaction:



Often tricalcium phosphate was precipitated in preference to hydroxyapatite because of its properties such as thermodynamic stability, higher insolubility and easy stabilization with  $\text{Mg}^{2+}$  (Stumm and Morgan, 1970). Dissolved carbon dioxide is considered important in phosphorus precipitation. Lime requirements are directly related to wastewater alkalinity and independent of phosphate concentration. Thus, some high alkalinity wastewaters need three times as much lime for effective precipitation (Shannon, 1980).

#### **2.2.2 Iron**

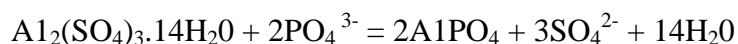
Iron(III) ions, commonly iron(III) chloride, are mainly responsible for phosphorus removal in wastewater treatment (Shannon, 1980). Iron(III) ions form strong complexes with pyrophosphate and tri-polyphosphates, which are removed by adsorption onto iron(III) hydroxophosphate surfaces (Jenkins *et al.*, 1971). The precipitation products for effective phosphorus removal depends on iron(III) dosage, phosphate concentration, temperature and pH (Kavanaugh

et al.,1978; Lijklema, 1980). The major advantages of using iron salts for precipitation are the low cost, and the sludges produced have excellent dewatering properties (Scott, 1973).

Two main reactions proceed simultaneously: (1) the formation and precipitation of ferric hydroxide and (2) formation and precipitation of ferric phosphates during precipitation of phosphate with ferric salts at pH 7. Phosphate removal is therefore the result of the kinetics of the two reactions which are pH dependent. In oxidizing environments, the phosphorus removal by ferrous salts will proceed with the following reactions simultaneously: the oxidation of ferrous ions to ferric ions; the hydrolysis of ferric ions by formation and precipitation of ferric hydroxide; the reaction of ferrous or ferric ions with phosphate to form ferric phosphate or ferrous phosphate which subsequently will be oxidized to ferric phosphate (Svanks, 1971).

### 2.2.3 Aluminium

The major form of chemicals used for precipitation is aluminium sulphate (alum), which undergoes the following reaction (Shannon, 1980):



The dosage of aluminium depends upon the concentration of soluble phosphate and colloidal particles. It includes aluminium reacting with the orthophosphate, followed by the destabilization of the organic material (Tenney and Stumm, 1965). Aluminium phosphate is formed because the precipitation is thermodynamically and kinetically favored over aluminium hydroxide formation (Jenkins et al., 1971). For effective removal, the alkalinity must be high enough to buffer the aluminium sulphate (Jenkins et al., 1971; Shannon, 1980). Aluminium precipitation is significantly affected by pH, calcium activity, the metal-to-phosphate dosage level and the bicarbonate activity of the water (Arvin and Petersen, 1980).

#### 2.2.4 Alternative chemical techniques

Various waste products such as waste pickle liquor (Azkona et al., 1979), fly ash (Gangoli and Thodos, 1974; Vinyard and Bates, 1979) and activated red mud from the aluminium industry (Shannon and Verghese, 1976) have been used for chemical phosphorus removal. Use of lime is proposed in conjunction with sea water for phosphorus removal (Ferguson and Vrale, 1984). Moreover, activated alumina could be used for phosphorus removal during wastewater treatment (Gangoli and Thodos, 1973; Bernhardt et al., 1981).

The chemical dose required for phosphorus removal is also influenced by effluent quality, wastewater characteristics (especially phosphorus concentrations, pH, total organic carbon, temperature and hardness), flow rate, hydraulic loading, chemical used, point and mode of application, and frequency of dose adjustment (Jenkins et al., 1971; Sutton et al., 1977; Wu, 1978; Shannon, 1980). The engineering aspects of the plant, such as mixing, flocculation and sedimentation characteristics, zonal residence time, internal floc shear, contact time, sludge age, and recycling dictate the requirements of chemicals.

No effect on anaerobic digestion due to chemical precipitation is well documented (Grigoropoulos et al., 1971; Jenkins et al., 1971; Boyko and Rupke, 1976). However, other authors have found that the use of chemicals for phosphorus precipitation does adversely affect anaerobic digestion. Adverse effects originated due to the toxicity of the chemicals, high pH, sulphate (acting as an alternative electron acceptor preventing true anaerobic conditions) and the lack of organic compounds (which reduces digestibility) (Van Fleet et al, 1974; Baillo et al., 1977; Dentel and Gossett, 1982).

### 2.2.5 Biological phosphorus removal

Biological P removal from wastewater can be achieved in two ways: stoichiometric coupling to microbial growth or enhanced storage in the biomass as polyphosphate (Poly-P) (Mino, T. et al, 1998). In a bio-P process, phosphorus is stored intracellularly by polyphosphate (poly-P) accumulating organisms (PAOs) when the wastewater is subjected to alternating anaerobic and aerobic conditions. Poly-P and glycogen provide energy under anaerobic conditions to allow for poly- $\beta$ -hydroxyalkanoates (PHA) storage which, in turns, provides energy and carbon for growth, poly-P storage and glycogen synthesis (Smolders *et al.*, 1994). The bacteria release orthophosphate under anaerobic conditions and under aerobic conditions they take up more orthophosphate than they released in the anaerobic zones (Batista and Jeong, 2006)

Enhanced biological phosphorous removal (EBPR) is becoming widely used as it produces effluents with very low phosphorus concentrations. Moreover, chemical phosphorus removal with metal ions has several disadvantages. The disadvantages include increased sludge production, chemical costs, and chemical feed control requirements.

Thus, current phosphorus removal methods rely on chemical precipitation or biological polyphosphate accumulation. Both of these methods are effective, but are not practical at all wastewater treatment facilities (e.g. biological accumulation needs specialized aerobic-anaerobic conditions that may not be present in all treatment systems). Biological  $\text{Fe}^{3+}$  reduction can lead to both accelerated carbon removal and increased phosphorus removal as vivianite in small scale wastewater treatment systems (septic tanks).

## 2.3 Phosphorus Removal as Vivianite

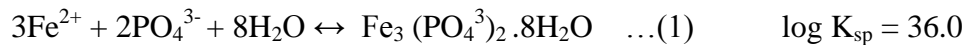
### 2.3.1 Mineral characteristics of vivianite

Vivianite is a hydrated iron phosphate mineral with the chemical formula  $\text{Fe}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}$  (Gaines et al., 1997). It occurs as either an amorphous mass or may crystallize as small encrustations or radiations of monoclinic crystals. The mineral structure of vivianite consists of  $\text{FeO}_2 \cdot 4\text{H}_2\text{O}$  octahedral and  $\text{Fe}_2\text{O}_6(\text{OH})_4$  octahedra linked together in uneven layers with  $\text{PO}_4$  tetrahedra connected between the layers via weak  $\text{H}_2\text{O}-\text{H}_2\text{O}$  hydroxyl bonds (Dixon and Weed, 1989).

The unit cells of crystal vivianite have dimensions of  $a = 10.08$ ,  $b = 13.43$ ,  $c = 4.70$ ,  $\beta = 104^\circ 30'$  (Mori, 1950). Pure vivianite has a specific gravity of 2.68. Vivianite is vulnerable to mechanical abrasion during transport. Fresh vivianite is colorless but alters to blue and then green with increasing exposure to oxygen (Dana, 1949). Vivianite requires specific conditions for its formation-sources of iron, phosphate, and water, as well as low levels of oxygen and sulfide. Microbial activity is also thought to play a part in vivianite formation (McGowan and Prangnell, 2006).

About 45% of the known phosphate minerals carry ferric or ferrous ions as a major component (Tomson and Vignona, 1984). Under aerobic conditions, phosphate salt precipitates as strengite ( $\text{FePO}_4 \cdot 2\text{H}_2\text{O}$ ) and under reducing conditions vivianite generally precipitates (Borno and Tomson, 1994). Basic mineral characteristics of vivianite are summarized in Table 01.

The molar ratio of  $\text{Fe}^{2+}$ -to- $\text{PO}_4^{3-}$  was 3:2 in accord with the stoichiometry given in Eq. (1), through which the  $\text{Fe}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}$  (vivianite) is formed:



(Jerome and Nriagu, 72; Liu and Zhao, 2007)

The temperature dependence of the negative logarithm of vivianite solubility ( $pK_{sp}$ ) product can be described between 5 to 90°C by  $pK_{sp} = -234.205 + 12,242.6/T + 92.510 \log T$ . (Borno and Tomson, 1994).

### 2.3.2 Phosphorus removal as vivianite

Vivianite ( $Fe_3(PO_4)_2 \cdot 8H_2O$ ) is an important sink for dissolved  $Fe^{2+}$  in phosphate-rich environments, and is considered as a very stable mineral due to its low solubility at neutral pH (Miot et al, 2009).  $Fe^{3+}$  oxides are subject to reduction (at least partially) under anaerobic conditions, which leads to release of soluble  $Fe^{2+}$  and can induce the precipitation of  $Fe^{2+}$ -bearing mineral phases (Sobolev and Roden, 2002).

**Table 1:** Mineral characteristics of vivianite

Property	Vivianite
Chemical formula	$Fe_3(PO_4)_2 \cdot 8H_2O$
Color	White or light gray, changing to blue or bluish-green on exposure to air
Crystals	Monoclinic Long prismatic to tabular
Luster	Vitreous Mother-of-Pearl luster on cleavage faces
Cleavage	Complete
Fracture	Bladed, fibrous, soft
Hardness (Mohs Scale)	1.5-2
Streak	Colorless, white, light blue, indigo, brownish, depending on degree of oxidation
Specific gravity	2.6-2.7
Solubility	Readily soluble in hydrochloric and nitric acids
Other	Thin crystal are flexible Vivianite is radioopaque on X-ray

Under most conditions, vivianite is the most important stable ferrous orthophosphate solid phase encountered in sediment environments (Nriagu, 1972). Vivianite has been shown to be stable under reducing conditions, high  $Fe^{2+}$  and  $PO_4^{2-}$  activities, and low sulphide activities (Rosenquist, 1972; Nriagu, 1972; Emerson and Widmer, 1978).



Phosphate co-precipitation (as vivianite) with calcite is an important mechanism controlling phosphorus concentrations in some hard water lakes (House, 2003). Particularly, vivianite ( $\text{Fe}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}$ ) is considered as one of the most important sinks of phosphorus in lakes (Nriagu and Dell, 1974; Buffe et al., 1989; Manning et al., 1991; Fortin et al., 1993; Viollier et al., 2000; Sapota et al., 2006).

In a wastewater system phosphorus solubility was directly related to the reduction of relatively insoluble ferric phosphate (Patrick, 1964). It is reported that vivianite is formed preferentially over struvite during anaerobic digestion (Carliell and Wheatley, 1997). The addition of iron salts to wastewater for phosphate removal results in the precipitation of ferric hydroxyphosphate and vivianite (Tomson and Vignona, 1984). If ferric phosphate is the phosphate precipitant, reduction of ferric iron to ferrous iron under anaerobic conditions would be expected to release phosphate to the digester supernatant (Mino, T. et al, 1998, Singer, 1970). No increase of phosphorus is observed in anaerobic digestion due to vivianite precipitation (Singer, 1970).

Further increases in iron concentrations in solutions already at equilibrium with vivianite (i.e., the effluent) would be expected to cause additional precipitation of vivianite. Vivianite precipitation is thought plausible in septic systems (Robertson et al, 1998) though no physical evidence has been reported yet.

### **2.3.3 Fe (III) reduction and mineral precipitation**

Microorganisms play an important role in the natural environment by determining the speciation of iron (Lemos et al, 2007). Microbial communities affect the pH and redox of natural waters and thus determine the form of the iron in solution, as well as the iron compounds that are precipitated (Brown et al, 1999). Dissimilatory iron reducing bacteria (DIRB) mediate the formation of iron mineral phases by delivering  $\text{Fe}^{2+}$  to the bulk solution after attachment to and

reduction of  $\text{Fe}^{3+}$  minerals at the cell surface (Arnold et al., 1986b; Lower et al., 2001), away from the cell via electron shuttling mechanisms (Lovley et al., 1996; Newman and Kolter, 2000), or possibly via soluble cytochromes (Seeliger et al., 1998).

Diverse microorganisms can obtain energy through dissimilatory respiration by transferring electrons from organic compounds to redox-active elements such as nitrogen, sulfur, or iron in their oxidized states (i.e., as  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$ , or  $\text{Fe}^{3+}$ ) in the absence of  $\text{O}_2$ . Human wastes contain a vast surfeit of reduced organic compounds, and most forms of nitrogen and sulfur in human wastes are also reduced, precluding their use as electron acceptors (Castillo-Gonzalez and Bruns, 2005). Iron-reduction is also the dominant redox process enabling the degradation of dissolved organic carbon (DOC), BTEX, naphthalene etc in the aquifer polluted by leachate from the Banisveld landfill in the Netherlands (Van Breukelen et al. 2004). Synthetic chelators such as  $\text{Fe}^{3+}$  EDTA and NTA played an important role in metal/radionuclide speciation, solubility, and mobility in surface waters and groundwater (Urrutia et al., 1999).

Several dissimilatory iron-reducing bacteria (DIRB) have been isolated from a variety of anoxic environments includes *Geobacter*, *Shewanella*, *Pelobacter*, *Geovibrio*, *Geospirillum*, *Ferrimonas*, *Geothrix*, *Desulfuromusa*, and *Desulfuromonas*, *Bacillus infernus*, *Thermoterrabacterium*, *Deferribacter thermophilus*, and *Thermoanaerobacter* spp. Also, there are several reports of enrichment cultures of bacteria that are capable of dissimilatory iron reduction (references cited in Kieft et al., 1999).

Both the dissolution and crystallization of iron minerals are function of environmental conditions. Solutes such as phosphate, organic anions and silicate strongly influence dissolution and nucleation behavior, as well as determine crystal habit (Cornell and Schwertmann, 2003;

Mann et al., 1989; Glasauer et al., 1999). Although the same basic processes of crystal nucleation and growth take place in both biotic and abiotic systems, the formation of minerals in the presence of DIRB will depend on the rate at which  $\text{Fe}^{2+}$  is released from the  $\text{Fe}^{3+}$  phase by bacterial reduction. This implies that the ability of bacteria to reduce  $\text{Fe}^{3+}$ , determined by the cells' physiologic status, can be manipulated to affect the mineral formed and its rate of crystallization (Glasauer et al, 2003).

The reactivity of the cell to metal ions at the bulk aqueous interface has several consequences. Sorption of metals to the outer membrane of gram-negative bacteria and subsequent mineral growth at the surface may compete with mineral formation in the bulk solution, or even disable the cells. Iron (hydr)oxide minerals added to cultures of *S. putrefaciens* have demonstrated high affinity for the cell surface (Glasauer et al., 2001). The location of bio-mineral precipitates will depend on properties of the metal and the microorganisms, reflecting chemical gradients established between bacteria and the extracellular environment.

Iron oxides formed in close association with bacteria (either as internal or external precipitates) are referred to as biogenic minerals. These minerals form as a result of the direct metabolic activity of bacteria or as a result of passive sorption and nucleation reactions. Passive iron sorption and nucleation onto bacterial cell walls represents another important mechanism leading to iron oxide formation (Fortin and Langley, 2005). Fixation of the metallic ions arises through interactions with anionic carboxyl or phosphoryl groups contained within the constituent homo- and heteropolymers of the bacterial cells (Doyle et al., 1980; Ferris and Beveridge, 1984, 1986). The molecular components in the envelopes of bacteria are particularly reactive and, consequently, metals tend to accumulate at the surface of the cells (Ferris and Beveridge, 1985).

$\text{Fe}^{2+}$  generated from iron oxide reduction can react with other surrounding ions to precipitate various iron bearing solid phases. Combined with sulfide it can form iron mono sulfide (Tsandev, 2005). This is postulated to be the preferential pathway (references cited in Tsandev, 2005). Moreover, it can combine with phosphate to produce vivianite minerals and combine with carbonate species can form siderite (Tsanev, 2005). If it does not precipitate, aqueous iron may complex to solids (including iron oxides and organic matter) by adsorption (Van Cappellen and Wang, 1996).

In presence of sulfide, iron phosphate interactions may be outcompeted by hydrogen sulfide (Roden and Edmonds, 1997). The presence of active sulfate-reducing bacteria may increase sulfide levels to the extent that vivianite is prevented from forming (Nriagu, 1972; Postma, 1977). Roden and Edmonds (1997) ruled out adsorption competition between phosphate and sulfate ions. They found that hydrogen sulfide could dissolve both amorphous and crystalline iron bearing solids to form iron sulfides. On the other hand,  $\text{Fe}^{3+}$  reduction can preclude the formation of sulfide from sulfate. Addition of amorphous iron<sup>3+</sup> oxyhydroxide to sediments in which sulfate reduction was the predominant terminal electron-accepting process inhibited sulfate reduction from 86 to 100% (Lovley and Phillips, 1987).

Nriagu (1972) conducted geochemical experiments to ascertain the stability of vivianite crystals under varying conditions of pH, Eh (redox potential), phosphate concentration, and sulfide concentration. These tests determined that in the system  $\text{Fe}_3(\text{PO}_4)_2\text{-H}_3\text{PO}_4\text{-H}_2\text{O-H}_2\text{S}$  at pH 7, vivianite exists in the low  $E_H$  range (−0.2 to −0.4) and at high to very high phosphate concentrations ( $\log \text{HPO}_4^{2-}$  of 0 to −5) (Nriagu, 1972). However, removal of  $\text{H}_2\text{S}$  from the system, could ensure stable and broader range of  $E_H$  spanning low to medium values (−0.07 to −0.36) for vivianite formation. This is also stable in a broader range of phosphate concentrations

from high to medium values ( $\log \text{HPO}_4^{2-}$  of 0 to -6) (Nriagu, 1972). Solubility constants for different  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  minerals have been tabulated in Table 02.

Vivianite is considered as the stable end product and the mean coherence length was influenced by the rate of  $\text{Fe}^{3+}$  reduction (Glasauer et al, 2003).  $^{57}\text{Fe}$  Massbauer data show that 43-67% of the total iron content of the sludges can be accounted for vivianite (Frossard et al, 1996). Phosphate removal has been reported in returned liquor of municipal wastewater

**Table 2:** Solubility constants of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  minerals

Metal	Mineral Name	Formula	Log $K_{sp}$
$\text{Fe}^{2+}$		$\text{Fe}(\text{OH})_2(\text{s})$	-15.9
	Siderite	$\text{FeCO}_3(\text{s})$	-10.55
	Vivianite	$\text{Fe}_3(\text{PO}_4)_2(\text{s})$	-36
		$\text{FeS}(\text{s})$	-16.84
	Wustite		11.688
$\text{Fe}^{3+}$	Ferrihydrite	$\text{Fe}(\text{OH})_3(\text{s})$	-37.11
	Goethite	$\alpha\text{-FeOOH}(\text{s})$	-41.5
	Lepidocrocite	$\gamma\text{-FeOOH}(\text{s})$	-46
	Hematite	$\alpha\text{-Fe}_2\text{O}_3(\text{s})$	-40.63

treatment using iron

reducing

bacteria (Ivanov et al., 2005).

Mixed culture is often very useful for degradation of complex compound. A co-culture of *Aeromonas veronii* and *Shewanella alga* shows better growth than when they are cultured separately. *A. veronii* produces formate, and possibly hydrogen, from citrate; the formate is then utilized as a substrate by *S. alga* which uses iron as an electron acceptor (Knight et al., 1996).

Possibly, the high concentrations of refractory organic material in the sediments affect the availability of iron. The pore water was calculated to be frequently supersaturated with respect to vivianite. This, however, did not seem to affect the phosphate concentrations in the pore water (Boers and Biles, 1991). Interstitial phosphate concentrations are influenced by

different processes: mineral forming reactions, microbial activity and transport (Boers and Biles, 1991). 1-2% of total iron and calcium can be present as a complex with dissolved organic carbon (DOC). It has been demonstrated in lakes (Olsson et al. 1997; Hupfer et al. 1998) and groundwater (Griffioen, 1994) that the formation of vivianite is partly controlled by the ratio of iron to phosphate. As long as the iron concentration exceeds sulfide formation, vivianite will be stable.

Thus, biological  $\text{Fe}^{3+}$  reduction can lead to precipitation of the completely insoluble mineral vivianite  $\text{Fe}_3(\text{PO}_4)_2 \cdot (\text{H}_2\text{O})_8$ , which would remain insoluble in secondary treatment sludge of onsite wastewater. It can be stimulated by adding bioavailable  $\text{Fe}^{3+}$  to promote  $\text{Fe}^{3+}$  reduction. Phosphate concentration dependent vivianite formation is favored at circumneutral pH or above. This is a simple amendment that can easily be “retrofitted” to any standard on-site wastewater (septic) system.

#### **2.3.4 Electron shuttling compounds (EES)**

Many microorganisms, e.g. iron-reducing, sulfate-reducing, and some fermenting bacteria, are capable of using humics (HS) as an electron acceptor for anaerobic oxidation of organic and inorganic electron donors (Lovley et al, 1996; Coates et al., 1998a; Benz et al., 1998; Cervantes et al., 2002). After microbial reduction, humics can transfer electrons to various  $\text{Fe}^{3+}$  oxide forms abiotically and recycle the humics to the oxidized form. Consequently it can then accept more electrons from the humics-reducing microorganisms (Lovley et al., 1996; 1998). Thus, in the presence of  $\text{Fe}^{3+}$ , each humic molecule may be reduced multiple times.

Quinone moieties in humics are important electron-accepting groups for microbial humics respiration (Scott et al., 1998). Reduced HS (containing the reduced form of quinones, i.e., hydroquinones) can transfer electrons to dissolved and solid-phase  $\text{Fe}^{3+}$ . AQDS serve as

good analogues for humic substances in microbial respiration (Loveley et al., 1999). Anthraquinone-2,6-disulfonate (AQDS), which contains quinone moieties that are believed to act in a manner similar to the semiquinones in humic substances, is a model compound that has been used frequently as a probe to study  $\text{Fe}^{3+}$  (hydr)oxide reductive dissolution by electron shuttling (Lovley et al., 1996; Coates et al., 1998b; Cervantes et al., 2003). Microorganisms transfer two electrons to AQDS, generating anthrahydroquinone-2,6-disulfonate (AHDS), which can then shuttle electrons to  $\text{Fe}^{3+}$ , thus regenerating AQDS (Lovley et al., 1996; Fredrickson et al., 1998).

Fermentative bacteria, nitrate- and sulfate-reducing bacteria, DIRB, and methanogens all are able to transfer electrons to AQDS (Bond and Lovley, 2002; Cervantes et al., 2002). These bacteria can reduce  $\text{Fe}^{3+}$  either by direct contact between the organism and the oxide surface or by indirect mechanisms not involving contact. These latter mechanisms include “electron shuttling” and soluble  $\text{Fe}^{3+}$  complexation with subsequent reduction (Lovley et al., 1996; Royer et al., 2002). When soil organic carbon content is significant, humic substances have been proposed to enhance  $\text{Fe}^{3+}$  reduction by serving as “electron shuttles” (i.e., biologically reducible kinetic intermediates) in extracellular electron-transfer processes between bacterial cells and  $\text{Fe}^{3+}$  (hydr)oxides (Lovley et al., 1996; Benz et al., 1998; Coates et al., 2002). Important candidates for the electron-accepting groups in humic substances are semiquinone moieties (Scott et al., 1998), complexed  $\text{Fe}^{3+}$  (Struyk and Sposito, 2001), and conjugated aromatic moieties (Chen et al., 2003).

Anthraquinone-2,6-disulfonate has been shown in laboratory studies to increase the reductive dissolution of synthetic or natural, amorphous (Fredrickson et al., 1998) or crystalline  $\text{Fe}^{3+}$  (hydr)oxides (goethite, hematite; Zachara et al., 1998). *Geobacter* species are of particular interest because of their capability of transferring electrons both to  $\text{Fe}^{3+}$  and to quinone moieties

present in HS (Lovley et al, 1996; Coates et al., 1998; Scott et al, 1998; Nevin et al, 2002). Additionally, several *Shewanella* strains were also shown to transfer electrons to natural organic matter or to anthraquinone-2,6-disulphonate (AQDS) (Chen et al, 2003; Newman and Kolter, 2000).



## CHAPTER 3 FERRIC IRON AMENDMENT INCREASES CARBON OXIDATION AND LIMITS METHANE PRODUCTION IN ON-SITE WASTEWATER (SEPTIC SYSTEMS)

### 3.1 Abstract

Onsite wastewater systems (OWSs), referred to as septic tanks, serve approximately 25% of the United States population; they are therefore a critical component of the total balance of carbon within natural water bodies. Septic systems typically operate under strictly anoxic conditions, and fermentation is the dominant process driving carbon transformation. Nitrate,  $\text{Fe}^{3+}$ , and sulfate reduction may be operating to a limited extent in any given septic tank. Electron acceptor amendments will increase carbon oxidation, but nitrate is toxic and sulfate generates corrosive sulfides, which may damage septic system infrastructure.  $\text{Fe}^{3+}$  reducing microorganisms transform all major classes of organic carbon that are dominant in septic wastewater: low molecular weight organic acids, carbohydrate monomers and polymers, and lipids.  $\text{Fe}^{3+}$  is not toxic, and the reduction product  $\text{Fe}^{2+}$  is minimally disruptive if the starting  $\text{Fe}^{3+}$  is added at a low to moderate concentration. We used carbon-14 radiolabeled acetate, lactate, propionate, butyrate, glucose, starch, and oleic acid to demonstrate that short and long term carbon oxidation is increased when different forms of  $\text{Fe}^{3+}$  are amended to septic wastewater. The rates of carbon mineralization to  $^{14}\text{CO}_2$  increased 2 to 5 times (relative to unamended systems) in the presence of  $\text{Fe}^{3+}$ . The extent of mineralization reached 90% for some carbon compounds when  $\text{Fe}^{3+}$  was present, compared to levels of 50-60% in the absence of  $\text{Fe}^{3+}$ .  $^{14}\text{CH}_4$  was not generated when  $\text{Fe}^{3+}$  was added, demonstrating that this strategy can limit methane emissions from septic systems. Moreover, iron reduction as terminal electron accepting process increases the microbial diversity of the wastewater. Amplified 16S rDNA restriction

analysis (ARDRA) confirmed that unique  $\text{Fe}^{3+}$ -reducing microbial communities increased significantly in iron-amended incubations. The form of  $\text{Fe}^{3+}$  added had a significant impact on the rate and extent of mineralization, with ferrihydrite and lepidocrocite favored as solid phase  $\text{Fe}^{3+}$  and chelated  $\text{Fe}^{3+}$  (with NTA or EDTA) as preferred soluble  $\text{Fe}^{3+}$  forms. Thus,  $\text{Fe}^{3+}$  amendment increased the efficiency of septic systems by opening a new metabolic niche and removing different forms of carbon to  $\text{CO}_2$ .

## 3.2 Introduction

Onsite wastewater treatment systems (OWSs), also referred to as septic systems, serve approximately 25% of the U.S. population (Conn et al, 2006, Lowe and Siegrist, 2008). Generally associated with “septic tanks,” on-site wastewater systems transform and treat human and household waste through complex physical, chemical, and biological processes (McCray and Christopherson, 2008). Advanced onsite wastewater treatment typically involves anaerobic septic tanks, followed by additional treatment units such as aerobic bioreactors, sand filters or constructed wetlands. Prefabricated septic tanks serve as a combined settling and skimming tank, an unmixed-unheated anaerobic digester and as a sludge storage tank (Crites and Tchobanoglous, 1998; Perkins, 1989; Parten, 2010). Settleable solids form a sludge layer at the bottom of the tank and flocculent materials such as oil and grease form a scum layer at the top of the aqueous portion of the tank.

Septic tank effluent is discharged from the clear space between the sludge and scum layer. The organic material retained in the bottom of the tank is decomposed anaerobically, and is converted to low molecular mass organic compounds and gases such as carbon dioxide ( $\text{CO}_2$ ), methane ( $\text{CH}_4$ ) and hydrogen sulfide ( $\text{H}_2\text{S}$ ). The settled sludge is decomposed biologically but often at an extremely slow rate that leads to sludge accumulation and a requirement for frequent cleaning (Crites and Tchobanoglous, 1998; Perkins, 1989; Parten, 2010). Dissolved organic carbon in the overlying aqueous and scum layers decomposes at different rates depending on the chemical composition of the incoming material (Crites and Tchobanoglous, 1998; Baumann et al, 1977).

While stable OWSs are effective for domestic wastewater they are relatively inefficient; converting only 30-50% of BOD<sub>5</sub> (Lowe et al., 2007) to mineralized end products such as CO<sub>2</sub> or CH<sub>4</sub> (Crites and Tchobanoglous, 1998; Perkins, 1989) within the tank. In addition, many systems generate more CH<sub>4</sub> than CO<sub>2</sub>, which is a concern because methane is a more potent greenhouse gas than carbon dioxide (US EPA, 2009). The carbon that does not get mineralized to either CH<sub>4</sub> or CO<sub>2</sub> ends up in: 1) the sludge layer, 2) the scum layer, or 3) the aqueous effluent that enters the natural environment (Crites and Tchobanoglous, 1998; Perkins, 1989).

The carbon in either the scum layer or the aqueous phase that enters the native environment can have a multitude of impacts ranging from benign to serious (Tchobanoglous et al., 2003, Peavy et al., 2003, McLean and Bledsoe, 1992; Dudal and Gerard, 2004; Coates et al., 2005). Generally speaking, excess exogenous carbon in natural environments is undesirable because of the capacity to deplete oxygen, mobilize metals, and generate nuisance odors (Tchobanoglous et al., 2003; Peavy et al., 2003, McLean and Bledsoe, 1992; Dudal and Gerard, 2004; Coates et al., 2005).

Shifting microbial metabolism to increase carbon oxidation can be accomplished by adding an appropriate electron acceptor, which increases the available free energy for carbon oxidation and opens new microbial niches in the selected environment (Coates et al., 2005; Castillo-Gonzalez et al., 2005). Nitrate (NO<sub>3</sub><sup>-</sup>) cannot be added to septic systems because it is a water hazard and the maximum contaminant level (MCL) for nitrate is 10mg/L, well below a concentration at which it would effectively increase carbon metabolism. Sulfate (SO<sub>4</sub><sup>2-</sup>) cannot be added because it generates sulfide(s) (S<sup>2-</sup>), which are extremely corrosive to septic system infrastructure. In addition, hydrogen sulfide gas (H<sub>2</sub>S) is extremely toxic in confined spaces like septic systems (Chaturvedi et al., 2001). The forms of nitrogen and sulfur entering septic tanks

(primarily as a result of human waste) are in the reduced valence state, which precludes their use as electron acceptors (Castillo-Gonzalez et al., 2005). Moreover, aerobic oxidation process needs additional treatment units and additional cost compare to anaerobic septic systems due to its complexity in design, maintenances, repair and presence of skilled person for proper functionality (Crites and Tchobanoglous, 1998; Perkins, 1989).

Ferric iron ( $\text{Fe}^{3+}$ ), alternatively, can be amended to septic systems without disrupting infrastructure (provided it is added at the appropriate concentration) and without any risk to human health or the environment. Moreover, reoxidation of  $\text{Fe}^{2+}$  is also possible (due to low level DO in incoming wastewater) that promotes  $\text{Fe}^{3+}$  use as an electron acceptor multiple times before permanent burial (Lovley, 2006).  $\text{Fe}^{2+}$  that leaves the septic system and flows into the sand filter, soil treatment unit, or native environment will also be re-oxidized to  $\text{Fe}^{3+}$ , where it can remain as an available electron acceptor. The resulting  $\text{Fe}^{3+}$  that can be generated downstream of the OWSs is benign to both human health and the OWSs infrastructure.

$\text{Fe}^{3+}$  reduction is one of the most important biogeochemical processes due to the abundance of ferric minerals (e.g., ferrihydrite, goethite, or phyllosilicate clay minerals) in soil and sedimentary environments, and its influence on the fate of organic or inorganic contaminants (Kostka et al., 2002; Roden et al., 2000; Zachara et al., 2001, Urrutia et al., 1998).  $\text{Fe}^{3+}$  reduction is mediated by  $\text{Fe}^{3+}$ -reducing microorganisms which are ubiquitous in subsurface environments (Coates et al., 1998, Coates et al., 1996b).

Septic tank effluent is known to contain many microorganisms (i.e. bacteria, archaea, viruses, and eukaryotes such as protozoa), both harmless and pathogenic (disease causing). At this time, little is known about the specific roles that specific microorganisms may play within the septic tank (Tomaras et al., 2007). On the other hand, iron-reduction is often the major

dominant redox process in subsurface settings polluted or amended with organic matter (Lovley et al. 2004).

$\text{Fe}^{3+}$  reduction is not a dominant reaction pathway in septic systems. Typically fermentation, methanogenesis, and perhaps sulfate reduction are the most prevalent reactions (Crites and Tchobanoglous, 1998). However,  $\text{Fe}^{3+}$  reducers are present and inactive (or with limited activity), and easily stimulated by adding  $\text{Fe}^{3+}$ .  $\text{Fe}^{3+}$  reducers can metabolize a broader array of carbon compounds than sulfate reducers, methanogens, or fermenters.  $\text{Fe}^{3+}$  reducers oxidize carbohydrates, lipids, and organic acids to  $\text{CO}_2$ , without accumulating intermediate carbon compounds (Coates et al., 1996a; Coates et al., 1998). Though iron can influence the color, odor and turbidity, the applied concentration will not change the color, odor and turbidity of wastewater. Moreover, iron is often found in wastewater which helps specific microbial growth. Additionally, there is wide use of ferric chloride in municipal wastewater system as coagulant. Therefore, adding  $\text{Fe}^{3+}$  to septic systems can increase the rate and extent of total carbon oxidation by allowing  $\text{Fe}^{3+}$  reducers to proliferate and become the dominant microbial community.

One additional benefit of  $\text{Fe}^{3+}$  reduction is that the organisms metabolize both acetate and  $\text{H}_2$  very rapidly, and maintain a low partial pressure of  $\text{H}_2$  in solution (0.2-0.8nM) (Chapelle et al, 1997). Methanogens require at least 5-30nM  $\text{H}_2$  in solution to continuously generate methane (Chapelle et al, 1997), or acetate as a precursor for aceticlastic methanogenesis (Lovley and Phillips, 1987a).  $\text{Fe}^{3+}$  reducers effectively inhibit both hydrogenotrophic and aceticlastic methanogenesis by maintaining the methane precursors ( $\text{H}_2$  and/or acetate) at concentrations too low to sustain methanogenic microbial populations (Lovley and Phillips, 1987a). Adding  $\text{Fe}^{3+}$  then has a twofold benefit for septic system operation – it simultaneously increases carbon

oxidation while decreasing methane production. There has been no reported publication about the activity of iron reducing bacteria in the anaerobic septic environment.  $\text{Fe}^{3+}$  amendment to septic systems essentially can open an “untapped” metabolic niche that allows greater carbon oxidation in a shorter time frame.

The purpose of this study was to quantify the increase in the rate and extent of carbon oxidation for several key carbon molecules when several different forms of  $\text{Fe}^{3+}$  were amended to onsite wastewater material. In addition, we quantified the proportion of carbon being mineralized to  $\text{CO}_2$  versus  $\text{CH}_4$  in all samples in the presence and absence of  $\text{Fe}^{3+}$ . In the majority of experiments  $\text{Fe}^{3+}$  greatly increased both the rate and extent of carbon oxidation to  $\text{CO}_2$ , and inhibited  $\text{CH}_4$  production. Iron reduction processes significantly increased iron reducing microorganisms and opened a new metabolic niche in onsite wastewater. These data can be used to develop an on-site wastewater treatment strategy using ferric iron amendments to promote more effective carbonaceous waste removal.

### 3.3 Materials and Methods

**3.3.1 Septic wastewater sampling and initial characterization:** Representative septic wastewater was collected in 2L pre-cleaned plastic jars from a single family household in Virden, IL in Dec, 2007. A “sludge judge” bailer (a long, hollow, clear plastic pole with a bottom-check-valve marked in 1-foot increments) was used to collect representative sample from flocculated and settled volume of septic sludge. Septic wastewater was sealed without a headspace with airtight closures, packed in ice chests and immediately transported to Newmark Civil Engineering Laboratory, Urbana, IL. Initial characterization of the water was performed immediately for pH, filtered and total COD,  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ ,  $\text{PO}_4^{3-}$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{SO}_4^{2-}$ , TSS, and VSS (Greenberg et al, 1992) before storing it at 4°C for further experiment and analysis.

**3.3.2 Batch  $^{14}\text{C}$  oxidation experiments:** 10 mL triplicate anaerobic samples of septic wastewater + 10 mM iron forms (six types) + 0.1 mL  $^{14}\text{C}$  carbon compounds (seven types) were incubated in 28 mL anaerobic pressure tubes (Bellco Glass, Inc.) with appropriate controls to quantify mineralization potential (Lovley et al., 1995) of the carbon compounds. 9 mL anaerobic liquid (wastewater) samples were prepared by sparging the medium tube with  $\text{N}_2\text{-CO}_2$  (80:20 [vol/vol]) for 6 minutes and flushing the headspace for 1 min. All transfers and incubations were performed under the same strict anoxic conditions (Cozzarelli et al, 2000). The tubes were sealed with thick butyl rubber stoppers and aluminum crimps (Bellco Glass, Inc.). All incubations were at 30 °C for 30 days.



10 mM concentration of Fe<sup>3+</sup> citrate, ferrihydrite (FeGel), lepidocrocite, Fe<sup>3+</sup> NTA, Fe<sup>3+</sup> EDTA and Fe<sup>3+</sup> phosphate were added to incubation tubes from stock iron solution before amendment of radiolabeled carbon. 0.1 µCi or 2.22 x 10<sup>5</sup> disintegrations per minute (dpm) of all individual [<sup>14</sup>C]-labeled carbon compounds were injected anoxically into the incubation tubes with a syringe and needle that had been flushed with N<sub>2</sub>. <sup>14</sup>C carbon compounds used were 2-<sup>14</sup>C-acetate (Moravek Biochemicals, Inc) , U-<sup>14</sup>C-lactate (Sigma Aldrich), 2-<sup>14</sup>C-butyrate (Moravek Biochemicals, Inc), 2-<sup>14</sup>C-propionate (Moravek Biochemicals, Inc), U-<sup>14</sup>C-glucose (Moravek Biochemicals, Inc), U-<sup>14</sup>C-starch (American Radiolabeled Chemicals, Inc) and 1-<sup>14</sup>C-oleic acid (lipid; Moravek Biochemicals, Inc).

One mL headspace gas samples were collected using 1 mL gas-tight syringe that had been flushed with anoxic gas to track <sup>14</sup>CO<sub>2</sub> (dpm) and <sup>14</sup>CH<sub>4</sub> (dpm) by separating using a gas chromatograph (GC) equipped with a gas proportional counter (GPC) (Model GC Ram: IN/US Corporation, Tampa, FL). Total production of <sup>14</sup>C-labeled inorganic carbon was calculated from the distribution of <sup>14</sup>CO<sub>2</sub> in the septic wastewater and the headspace, as determined by adding H<sup>14</sup>CO<sub>3</sub><sup>-</sup> standards to the septic samples using a scintillation counter. The proportion of H<sup>14</sup>CO<sub>3</sub><sup>-</sup> versus <sup>14</sup>CO<sub>2</sub> arising from H<sup>14</sup>CO<sub>3</sub><sup>-</sup> was quantified at each time point to calculate a pH dependent ‘partition’ value, which was used for measuring percent (%) mineralization of each of the <sup>14</sup>C carbon compounds. The mineralization equation is:

$$\text{Partition} = (Y_{\text{HCO}_3^-} * (V_{\text{head space}} / V_{\text{sample}})) / Z_{\text{HCO}_3^-}$$

$$\% \text{ of } [^{14}\text{C}]\text{-compounds recovered as } ^{14}\text{CO}_2 \text{ or } ^{14}\text{CH}_4$$

$$= (Y_{[^{14}\text{C}] \text{ compound}} \cdot V_{\text{head space}} / \text{partition}) / (Z_{[^{14}\text{C}] \text{ compound}})$$

here,  $Y_{\text{HCO}_3}$  = radioactivity of  $\text{CO}_2$  measured in the head space of  $\text{H}^{14}\text{CO}_3^-$  amended sample (dpm),  $Z_{\text{HCO}_3}$  = total radioactivity of  $\text{H}^{14}\text{CO}_3^-$  amended to the  $\text{H}^{14}\text{CO}_3^-$  amended sample (dpm),  $Y_{[^{14}\text{C}] \text{ compound}}$  = radioactivity of  $\text{CO}_2/\text{CH}_4$  measured in the head space of  $[^{14}\text{C}]$  - amended sample (dpm/mL),  $Z_{[^{14}\text{C}] \text{ compound}}$  = total radioactivity of  $[^{14}\text{C}]$  compound amended to the  $[^{14}\text{C}]$  - amended sample (dpm),  $V_{\text{head space}}$  = total volume of the head space of the anaerobic tube (mL),  $V_{\text{sample}}$  = volume of sample injected in GC-GPC for radioactivity measurement.  $^{14}\text{CH}_4$  does not require a partition value, as we assumed aqueous saturation and equilibrium within the headspace.

### 3.3.3 Molecular methods:

**3.3.3a Extraction of nucleic acids and PCR amplification:** Representative septic sludge from one of the triplicates of the iron amended bottles and un-amended incubations were collected at the start and end of the experiment. Samples were kept frozen at  $-80^\circ \text{C}$  until the experiments were completed. Deoxyribonucleic acid (DNA) extraction was performed using the FastDNA SPIN Kit (MP Biomedicals) that involves cell lysis by bead-beating, DNA isolation, and purification. The extracted partial 16S ribosomal ribonucleic acid (rRNA) genes were amplified by *polymerase chain reaction* (PCR) with universal bacterial primer pair 338F (50-ACT CCT ACG GGA GGC AGC-30) and 907R (50-CCG TCA ATT CCT TTG AGT TT-30). The quality and band pattern of PCR products were checked by electrophoresis on 2% agarose gel.

**3.3.3b Construction of clone libraries and sequencing:** Clone libraries were constructed using the 16S rRNA gene PCR products and amplified ribosomal DNA restriction analysis (ARDRA) was performed (Wei and Finneran, 2009; Wei and Finneran, 2011). TOPO TA Cloning Kit for sequencing with One Shot Mach1-T1 competent cells (Invitrogen) was used for

cloning. Fifty clones were selected randomly from each clone library and were re-amplified using the vector-specific primers (0.25  $\mu$ M each) M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') (10  $\mu$ M, Invitrogen). The plasmid inserts were digested with restriction endonucleases MspI and HhaI (NE BioLabs). The resulting band fragments were visualized and recorded using the Luminary Fx. 2.0-FOTO/Analyst Fx. System (Fotodyne Inc., Hartland, WI). Representative clones of unique ARDRA patterns of the selected 50 clones were sequenced at W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois, Urbana-Champaign.

**3.3.3c Microbial community and phylogenetic analysis:** The partial 16S rRNA gene sequences of individual clones were compared with GenBank databases using nucleotide BLAST (National Center for Biotechnology Information). Supplemental information was cross-checked using Ribosomal Database project (RDP) (Michigan State University) and Greengenes database to improve the accuracy. Checks for chimeric sequences were performed with Chimera Check software on the Greengenes database. Operational taxonomic units (OTUs) were determined by grouping distinct clones of each type of incubation. Shannon index (H) and Simpson's indexes (D) of diversity were calculated to assess the diversity of microbial communities due to iron amendment (Legendre and Legendre, 1998). Both the number of OTUs and each OTU's relative abundance in the whole clone library were taken into consideration for the two statistical parameters. The following equations were used:

$$H = \left\{ - \sum_{i=1}^S (p_i \ln p_i) \right\} - \{(S - 1)/2N\}$$

$$D = 1 - \sum_{i=1}^S p_i^2$$

Here, S designates number of OTUs, N designates total number of all clones (i.e., 50 for each clone library), and  $p_i$  stands for relative abundance of each OTU- calculated as the ratio of the number of clones in each distinct clone group to the total number of clones in the community.

Jaccard similarity coefficients (J) were calculated to estimate the similarity of the microbial communities between initial and final time points of the experiment (Legendre and Legendre, 1998). The following equation was used:

$$J = W / (a_{T=0} + a_{T=30} - W)$$

W is the number of OTUs shared between microbial communities of T = 0 and 30 days and  $a_{T=0}$  and  $a_{T=30}$  are the total numbers of OTUs in each of the communities.

Dominant clones (operationally defined as 5% or higher) present in all the samples were used for the construction of phylogenetic tree using MEGA5 software (Tamura et al., 2011). Typical known anaerobic wastewater  $\text{Fe}^{3+}$ -reducing bacteria, sulfate reducing bacteria, and nitrate reducing bacteria were chosen as reference microorganisms. Multiple sequence alignment was conducted using the MUSCLE algorithm in MEGA5 (Edgar, 2004). The tree was obtained using the Neighbor-Joining method (Saitou and Nei, 1987) and genetic distances were estimated by using the maximum composite likelihood method estimated by the Tamura-Nei model (Tamura, 1993). The confidence of phylogeny was tested by bootstrap resampling for 1000 replicates (Felsenstein, 1985). The sequences reported in this study have been deposited in GenBank.

**3.3.4 Analytical methods:** Conventional wastewater parameters including alkalinity, ammonia, filtered/total chemical oxygen demand (COD), and total/volatile suspended solids were analyzed

by *Standard Methods* (Greenberg et al., 1992). The aqueous phase pH was measured using semi micro pH probe (Thermo Scientific Inc., Pittsburgh, PA). Total iron (ferric and ferrous) in the solid phase and soluble  $\text{Fe}^{2+}$  were quantified using the ferrozine assay (Stookey, 1970). Briefly, total iron was extracted in 0.5N HCl and the resulting extract was chelated with Ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine);  $\text{Fe}^{2+}$  forms a strong purple color which can be determined at 562 nm. The  $\text{Fe}^{3+}$  portion of the extract was reduced with 6.25N hydroxylamine, and the  $\text{Fe}^{3+}$  portion was determined by difference (initial ferrous minus final ferrous). Aqueous  $\text{Fe}^{2+}$  was quantified using the Ferrozine assay minus the second step.  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{SO}_4^{2-}$  and  $\text{PO}_4^{3-}$  were analyzed using an Ion Chromatography (IC) (DX-1000 ion chromatograph, Dionex Corp.) using 0.1 mL of filtered sample. Quantification of  $^{14}\text{CO}_2$  and  $^{14}\text{CH}_4$  was described above.

## 3.4 Results

### 3.4.1 Characterization of septic system material

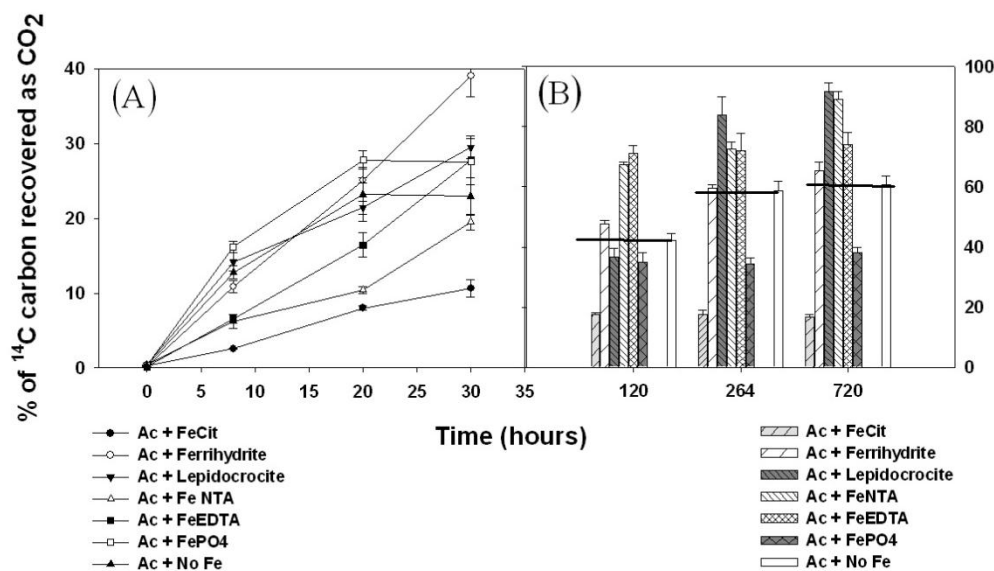
The septic material included both water and sludge, and was homogenized by shaking prior to analyses. All aqueous analytes were filtered. Samples for analysis of total bioavailable iron, total suspended solids, and total COD were not filtered. The pH of the septic material was 6.6. The total starting COD was 579mg/L; the soluble COD (filtered) fraction was 354mg/L of the wastewater supernatant. Total suspended solids were 2177mg/L, and volatile suspended solids were 1240mg/L. Starting phosphorus was 9.5mg/L. The ferric iron concentration was 6.7mg/L, which was due to the suspended solids in the septic material. Aqueous ferrous iron was 21.3mg/L. Nitrate and ammonium were below detection; nitrite was 3.3mg/L. Sulfate was 17mg/L.

### 3.4.2 Oxidation of $^{14}\text{C}$ -labeled carbon compounds and inhibition of methane production in $\text{Fe}^{3+}$ -amended septic material

Seven different  $^{14}\text{C}$ -radiolabeled carbon molecules were used to quantify mineralization changes in  $\text{Fe}^{3+}$ -amended septic material: acetate, lactate, butyrate, propionate, starch, glucose, and oleic acid (a model lipid). This matrix compared key carbon intermediates (the organic acids), carbohydrate polymers and monomers (starch and glucose), and fats (oleic acid). These are primary carbon compounds encountered in septic environments that contribute to soluble and total COD, as well as methane (Crites and Tchobanoglous, 1998). The  $\text{Fe}^{3+}$  forms used were: ferric citrate, ferrihydrite (referred to as Fe Gel), lepidocrocite, ferric NTA, ferric EDTA, and ferric phosphate. Mineralization of seven different  $^{14}\text{C}$ -radiolabeled carbon molecules was monitored over the short term (30 hours), and long term (120 to 720 hours). Nitrate and sulfate were also amended for comparison (data not shown). 30-40 percent of ferric iron added initially

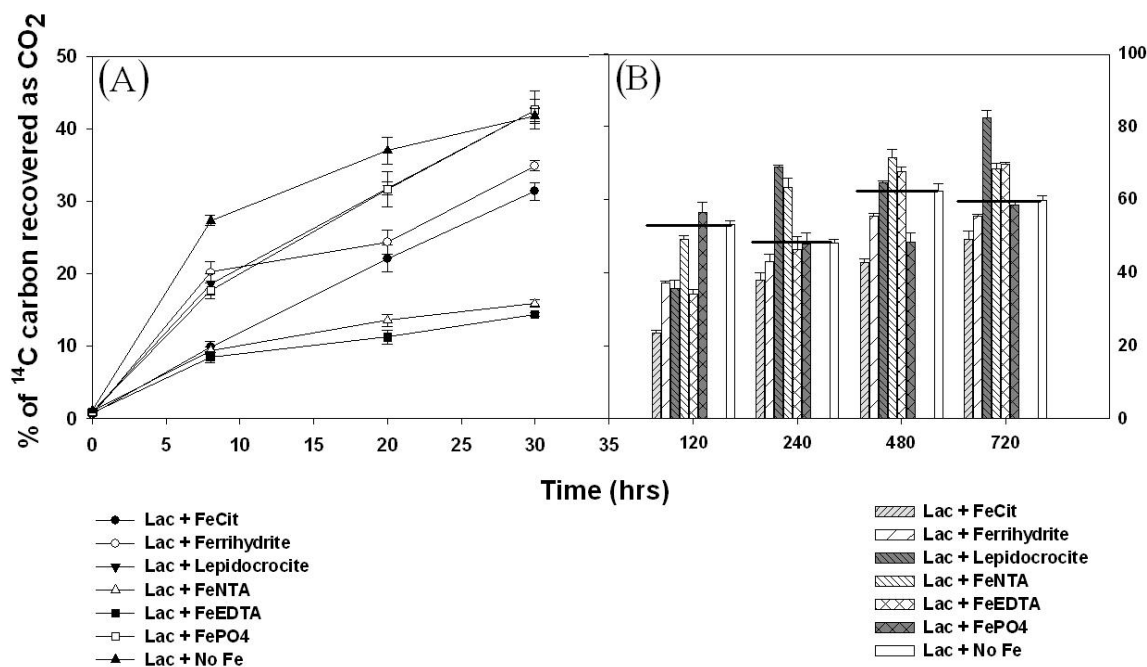
was reduced to ferrous iron within 30 hrs. A significant fraction of ferric iron was converted to ferrous iron within 264 hrs period of the experiment.

**3.4.2a Acetate.** Short term mineralization of acetate was most significantly enhanced by ferrihydrite (Figure 3a). Lepidocrocite, ferric EDTA, and ferric phosphate also enhanced short term mineralization relative to un-amended septic material. Ferric citrate decreased mineralization (Figure 3a). Long term acetate mineralization was increased by ferrihydrite, lepidocrocite, ferric NTA and ferric EDTA relative to un-amended samples (Figure 3b). The maximum mineralization of acetate was approximately 90% in ferric NTA and lepidocrocite amended samples. Ferric EDTA maximum mineralization was 75% whereas maximum mineralization in unamended samples was 60%. Alternatively, mineralization as methane was less than 0.6% up to 720 hours in all  $\text{Fe}^{3+}$  amended bottles, whereas un-amended bottles had as much as 2.4% methane in the same time period (Table 3).



**Figure 3:**  $^{14}\text{C}$  acetate recovered as  $\text{CO}_2$  in  $\text{Fe}^{3+}$  amended and un-amended septic wastewater. Results are the means of triplicate analyses; bars indicate one standard deviation. 10 mL triplicate anaerobic samples of septic wastewater + 10 mM iron forms (six types) + 0.1 mL  $^{14}\text{C}$  acetate were incubated in 28 mL anaerobic pressure tubes.

**3.4.2b Lactate.** Short term mineralization of lactate as CO<sub>2</sub> was not significantly enhanced by the Fe<sup>3+</sup> amendments. Ferric NTA and ferric EDTA decreased short term mineralization (Figure 4a). Long term lactate mineralization as CO<sub>2</sub> was increased by lepidocrocite, with ~85% being the maximum extent of lactate mineralization. Ferric EDTA and ferric NTA averaged 70% mineralization compared to 62% mineralization for unamended system. Methane production was significant over the long term in lactate amended incubations that were unamended or were amended with sulfate; 3% of the lactate was recovered as methane in the sulfate amended incubations and 5% of the lactate was recovered as methane in the unamended incubations (Table 3). Approximately 2.15% of the lactate was recovered as methane in lepidocrocite amended incubations; all remaining Fe<sup>3+</sup> amended bottles were 0.75% or less (Table 3).



**Figure 4:** <sup>14</sup>C lactate recovered as CO<sub>2</sub> in Fe<sup>3+</sup> amended and un-amended septic wastewater. Results are the means of triplicate analyses; bars indicate one standard deviation. 10 mL triplicate anaerobic samples of septic wastewater + 10 mM iron forms (six types) + 0.1 mL <sup>14</sup>C lactate were incubated in 28 mL anaerobic pressure tubes.



**3.4.2c Propionate.** Short term mineralization of propionate was relatively low (approx. 5%), but ferric NTA and ferric EDTA increased the extent of mineralization within 120 hours by 25-60% compared to unamended septic material (Figure 5a and Figure 5b). Both chelated ferric iron forms stimulated long term propionate mineralization, and the highest  $\text{Fe}^{3+}$  amended propionate mineralization quantified was ~80% in 720 hours (ferric NTA amendment). Comparable unamended material had a high of <5% (Figure 5b). Propionate conversion to methane was negligible (<1%) in all treatments (Table 3).

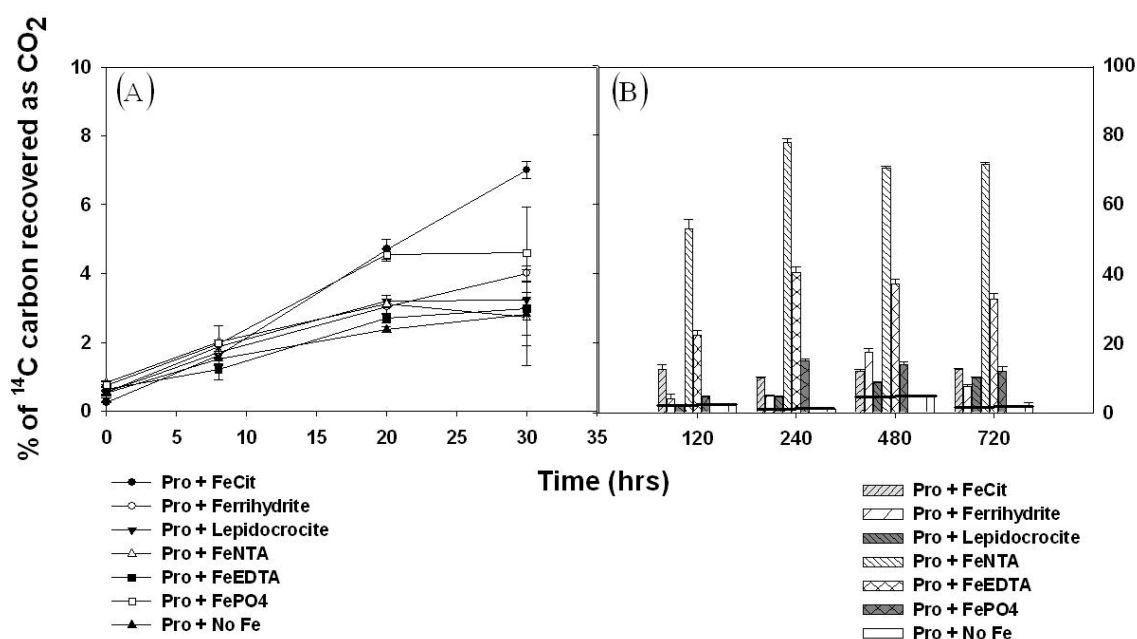
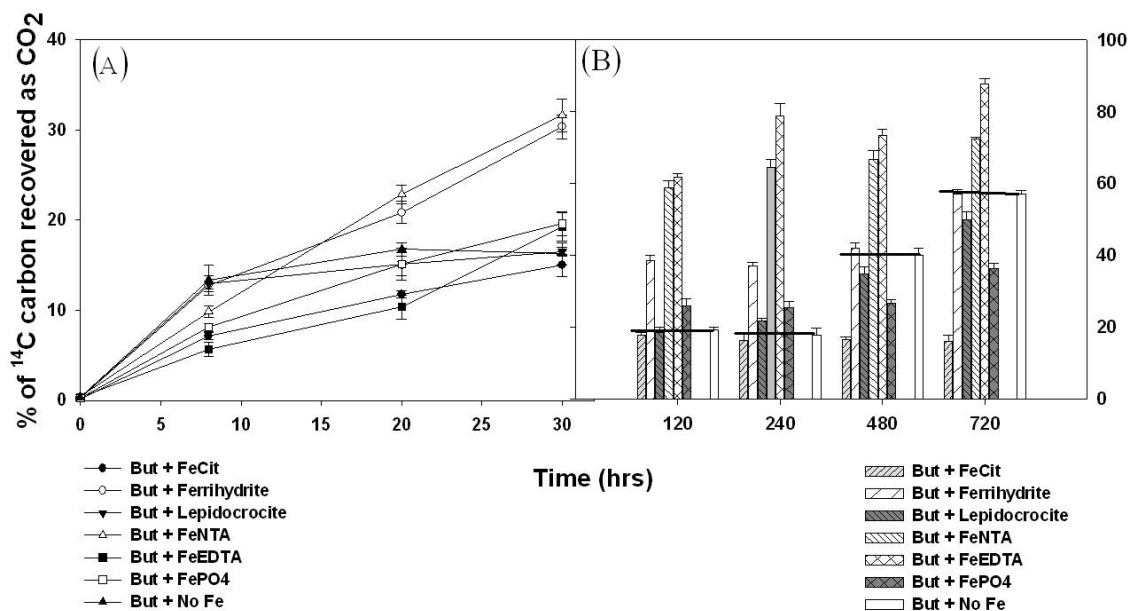


Figure 5:  $^{14}\text{C}$  propionate recovered as  $\text{CO}_2$  in  $\text{Fe}^{3+}$  amended and un-amended septic wastewater. Results are the means of triplicate analyses; bars indicate one standard deviation. 10 mL triplicate anaerobic samples of septic wastewater + 10 mM iron forms (six types) + 0.1 mL  $^{14}\text{C}$  propionate were incubated in 28 mL anaerobic pressure tubes.

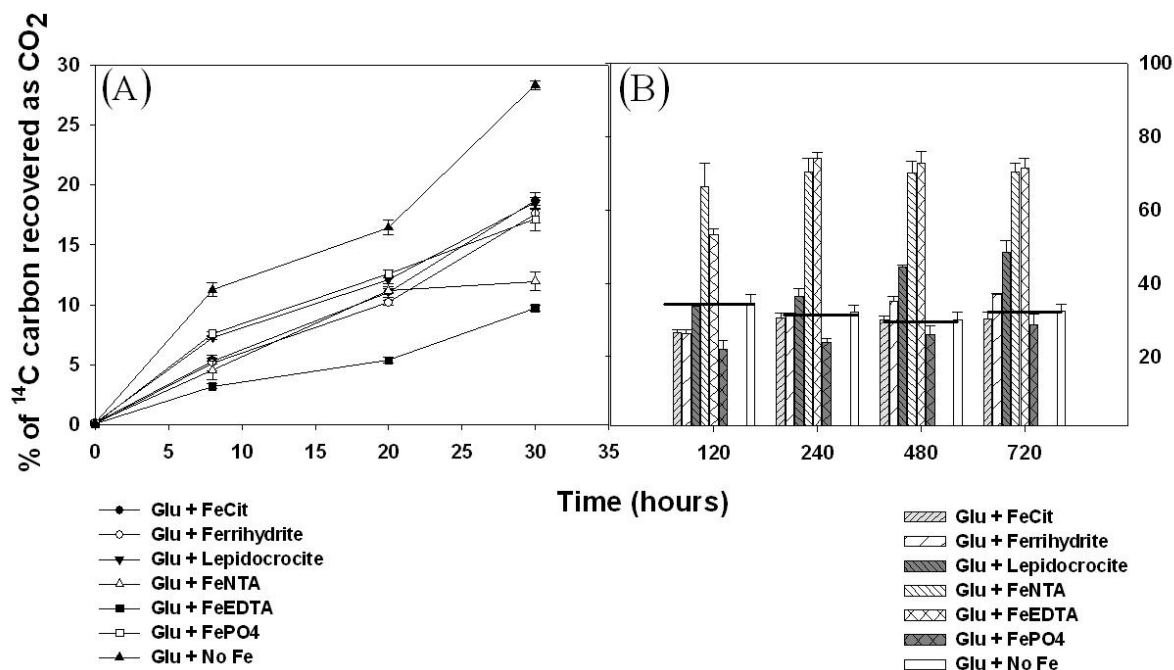
**3.4.2d Butyrate.** Short term butyrate mineralization was increased by ferrihydrite and ferric NTA; both increased mineralization relative to unamended incubations by 10% or more (Figure 6a). Several  $\text{Fe}^{3+}$  amendments increased butyrate mineralization over the long term. Ferrihydrite, ferric NTA and ferric EDTA all stimulated mineralization relative to unamended incubations (Figure 6b.) The maximum mineralization was ~87%, with ferric EDTA

amendment. Methane production in unamended incubations reached 4.7% after 720 hours (Table 3). The material amended with lepidocrocite had 3% methane within the same time frame; all remaining  $\text{Fe}^{3+}$  amended bottles were 0.65% or less (Table 3).



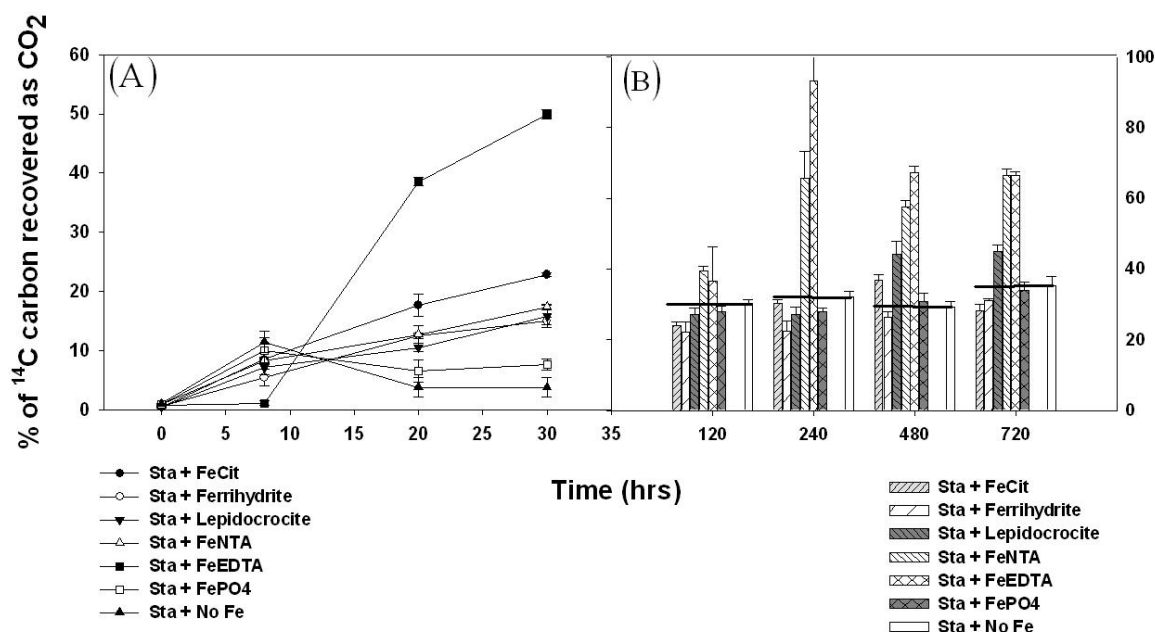
**Figure 6:**  $^{14}\text{C}$  butyrate recovered as  $\text{CO}_2$  in  $\text{Fe}^{3+}$  amended and un-amended septic wastewater. Results are the means of triplicate analyses; bars indicate one standard deviation. 10 mL triplicate anaerobic samples of septic wastewater + 10 mM iron forms (six types) + 0.1 mL  $^{14}\text{C}$  butyrate were incubated in 28 mL anaerobic pressure tubes.

**3.4.2e Glucose.** Glucose was mineralized most readily in the unamended incubations within the first 30 hours, with 30% being converted to  $\text{CO}_2$  and negligible methane production (Figures 7a and 7b). Ferric EDTA limited mineralization in the short term relative to other  $\text{Fe}^{3+}$  amendments (Figure 7a). Ferric NTA and ferric EDTA stimulated glucose mineralization over the long term, with approximately 70% mineralized to  $\text{CO}_2$ , relative to a high of 33% for the unamended incubations (Figure 7b). Methane was negligible in all glucose-amended septic material (approximately 1% or less; Table 3).



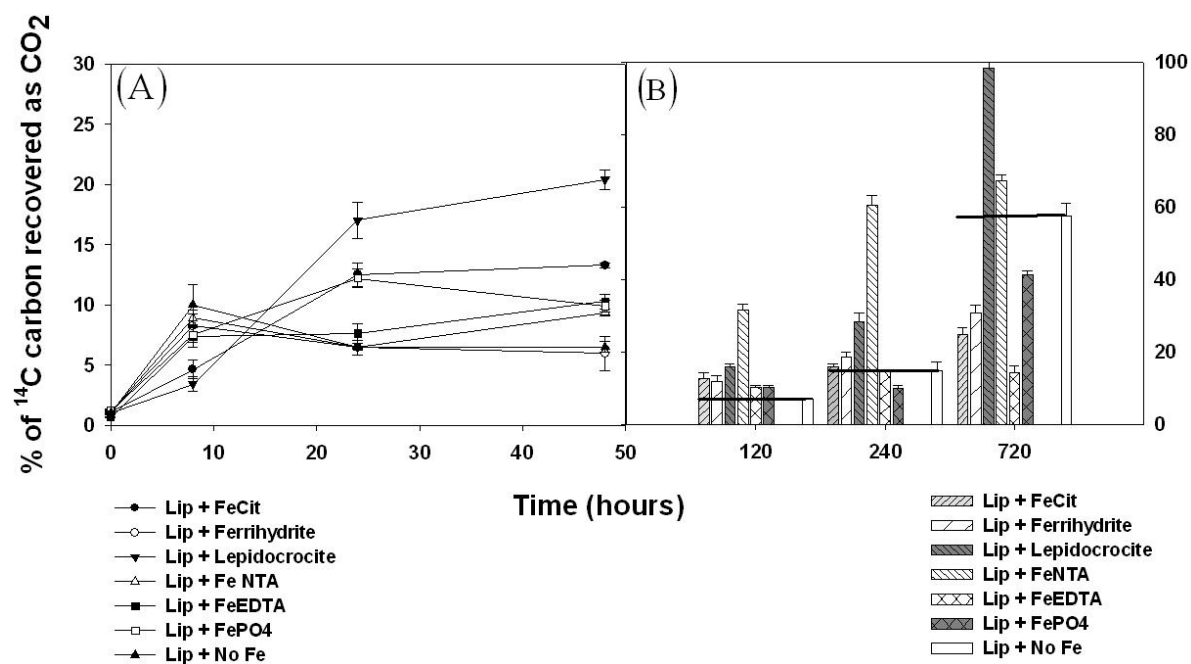
**Figure 7:**  $^{14}\text{C}$  glucose recovered as  $\text{CO}_2$  in  $\text{Fe}^{3+}$  amended and un-amended septic wastewater. Results are the means of triplicate analyses; bars indicate one standard deviation. 10 mL triplicate anaerobic samples of septic wastewater + 10 mM iron forms (six types) + 0.1 mL  $^{14}\text{C}$  glucose were incubated in 28 mL anaerobic pressure tubes.

**3.4.2f Starch.** Short term starch mineralization was significantly increased by ferric EDTA (Figure 8a). Over the long term; ferric EDTA increased mineralization to the greatest extent, with 93% of the starch being mineralized, as compared to 35% in the unamended incubations (Figure 8b). Methane production was between 0.6 and 1.4% in all treatments (Table 3).



**Figure 8:**  $^{14}\text{C}$  starch recovered as  $\text{CO}_2$  in  $\text{Fe}^{3+}$  amended and un-amended septic wastewater. Results are the means of triplicate analyses; bars indicate one standard deviation. 10 mL triplicate anaerobic samples of septic wastewater + 10 mM iron forms (six types) + 0.1 mL  $^{14}\text{C}$  starch were incubated in 28 mL anaerobic pressure tubes.

**3.4.2g Lipid.** Oleic acid mineralization was stimulated in the short term by lepidocrocite (Figure 9a). Lepidocrocite also stimulated lipid mineralization over the long term, with approximately 98% of the lipid carbon recovered as  $\text{CO}_2$ . Lipid mineralization in unamended incubations was 58% in the same time frame. Ferric NTA also promoted higher mineralization than unamended incubations (67% versus 58%) over the long term. Methane production reached 7% in the unamended bottles, but also reached 5% in lepidocrocite amended bottles at the same time point (Table 3).



**Figure 9:**  $^{14}\text{C}$  lipid recovered as  $\text{CO}_2$  in  $\text{Fe}^{3+}$  amended and un-amended septic wastewater. Results are the means of triplicate analyses; bars indicate one standard deviation. 10 mL triplicate anaerobic samples of septic wastewater + 10 mM iron forms (six types) + 0.1 mL  $^{14}\text{C}$  lipid were incubated in 28 mL anaerobic pressure tubes.

**Table 3a:** Maximum % of  $^{14}\text{C}$  carbon molecules recovered as  $\text{CO}_2$

Iron Forms	Maximum % of $^{14}\text{C}$ Carbon Oxidized to $^{14}\text{CO}_2$													
	Acetate		Glucose		Lactate		Propionate		Butyrate		Starch		Lipid	
	$\text{CO}_2$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CO}_2$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CO}_2$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CO}_2$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CO}_2$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CO}_2$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CO}_2$ (SD <sup>1</sup> )	H <sup>2</sup>
<b>FeCitrate</b>	17.47 (0.74)	No	30.26 (1.86)	No	49.13 (2.27)	No	12.56 (0.27)	Yes	17.89 (0.56)	No	36.75 (1.68)	No	24.77 (1.98)	No
<b>FeGel</b>	65.41 (2.76)	No	36.88 (0.31)	Yes	55.53 (0.37)	No	17.51 (1.01)	Yes	57.2 (1.18)	No	30.97 (0.61)	No	30.76 (2.11)	No
<b>Lepidocrocite</b>	91.61 (3.08)	Yes	48.41 (3.36)	Yes	82.43 (1.98)	Yes	9.99 (0.34)	Yes	49.91 (2.12)	No	44.87 (1.96)	Yes	98.37 (1.77)	Yes
<b>Fe<sup>3+</sup> NTA</b>	89.01 (2.8)	Yes	70.47 (2.25)	Yes	71.46 (2.13)	Yes	77.73 (1.23)	Yes	72.4 (0.62)	Yes	66.38 (1.97)	Yes	67.31 (1.63)	Yes
<b>Fe<sup>3+</sup> EDTA</b>	74.19 (3.88)	Yes	74.22 (1.52)	Yes	69.57 (0.58)	Yes	40.33 (1.78)	Yes	87.62 (1.55)	Yes	93.04 (6.05)	Yes	14.43 (1.86)	No
<b>Fe<sup>3+</sup> Pyrophosphate</b>	38.2 (1.55)	No	28.58 (3.02)	No	58.41 (0.83)	No	14.9 (0.69)	Yes	36.29 (1.52)	No	34.03 (2.26)	No	41.22 (1.12)	No
<b>Un-Amended System</b>	60.73 (2.78)	N/A	32.47 (1.95)	N/A	62.23 (2.06)	N/A	4.52 (0.25)	N/A	57.08 (0.90)	N/A	35.12 (2.68)	N/A	57.48 (3.45)	N/A

\*1: SD = Standard Deviation

\*2: H = Hypothesis that oxidation to  $\text{CO}_2$  due to iron amendment is significantly greater than no iron amendment oxidation (95% confidence interval: using *t*-Test: Two-sample assuming unequal variances)

**Table 3b:** Maximum % of  $^{14}\text{C}$  carbon molecules recovered as  $\text{CH}_4$

Iron Forms	Maximum % of $^{14}\text{C}$ Carbon Mineralized to $^{14}\text{CH}_4$													
	Acetate		Glucose		Lactate		Propionate		Butyrate		Starch		Lipid	
	$\text{CH}_4$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CH}_4$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CH}_4$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CH}_4$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CH}_4$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CH}_4$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CH}_4$ (SD <sup>1</sup> )	H <sup>2</sup>
<b>FeCitrate</b>	0.59 (0.25)	Yes	0.2 (0.07)	No	0.59 (0.13)	Yes	0.4 (0.07)	No	0.53 (0.19)	Yes	0.69 (0.13)	No	1.00 (0.52)	Yes
<b>FeGel</b>	0.44 (0.14)	Yes	0.36 (0.23)	No	0.61 (0.40)	Yes	0.51 (0.17)	No	0.57 (0.31)	Yes	0.75 (0.20)	No	1.39 (0.91)	Yes
<b>Lepidocrocite</b>	0.5 (0.05)	Yes	0.14 (0.07)	No	2.15 (0.24)	Yes	0.97 (0.46)	No	2.97 (0.37)	Yes	0.65 (0.35)	No	4.92 (0.33)	Yes
<b>Fe<sup>3+</sup> NTA</b>	0.36 (0.07)	Yes	1.29 (0.60)	No	0.70 (0.29)	Yes	0.64 (0.11)	No	0.55 (0.22)	Yes	0.53 (0.25)	No	1.99 (0.64)	Yes
<b>Fe<sup>3+</sup> EDTA</b>	0.41 (0.16)	Yes	0.17 (0.06)	No	0.57 (0.25)	Yes	0.53 (0.11)	No	0.66 (0.18)	Yes	1.36 (0.80)	No	1.24 (0.17)	Yes
<b>Fe<sup>3+</sup> Pyrophosphate</b>	0.46 (0.30)	Yes	0.15 (0.03)	No	0.65 (0.40)	Yes	0.68 (0.65)	No	0.63 (0.02)	Yes	0.67 (0.37)	No	1.41 (0.03)	Yes
<b>Un-Amended System</b>	2.44 (0.62)	N/A	0.17 (0.10)	N/A	4.72 (0.76)	N/A	0.37 (0.15)	N/A	4.62 (0.25)	N/A	0.68 (0.17)	N/A	6.59 (0.65)	N/A

\*1: SD = Standard Deviation

\*2: H = Hypothesis that mineralization to  $\text{CH}_4$  due to no iron amendment is significantly greater than iron amendment mineralization (95% confidence interval: using t-Test: Two-sample assuming unequal variances)

### 3.4.3 Microbial diversity changes due to Fe<sup>3+</sup> amendment and dominant microorganisms

The onsite wastewater system is rich in microbial diversity. There were 74 distinct clone groups identified in the iron amended and un-amended wastewater. The clone groups (OTUs) were given alphanumeric names from HA1 to HA74, and the results from GenBank database alignments are summarized in Table 7 (Appendix). The microbial population abundance for each incubation is depicted by the phylotype frequency in the clone library, as illustrated in Fig. 30 (Appendix). Percent distribution of known and likely Fe<sup>3+</sup> reducers at time = 0 and time = 30 days for samples with iron amendment and un-amended sample were shown in figures 10, 11, 12 and 13.

One interesting characteristic was no specific microorganisms dominated the incubation due to presence of wide variety of microorganisms. Known iron reducing microorganism was reduced in un-amended incubations from 31% to 27.5% at the end of experiment and likely iron reducers changed from 7.2% to 7.5% (Fig. 10). The diversity of microorganisms did not change noticeably during the experimental period confirmed by Shannon index and Simpson's index of diversity ( $H_{T=0} = 2.208$ ,  $H_{T=30} = 2.399$ ,  $D_{T=0} = 0.897$ ,  $D_{T=30} = 0.9025$ ) (Table 04). The dominant known iron reducing microorganisms found in this batch of experiment were Chlorobi group bacterium clone D25\_44 ( $\beta$ -Proteobacteria), uncultured Acidobacteriaceae bacterium clone D12\_13 (Firmicutes) and uncultured bacterium clone MS044 (Firmicutes). Microbial diversity increased significantly in iron amended incubations. Both known iron reducers and likely iron reducers biomass increased significantly for ferrihydrite, lepidocrocite and iron-EDTA amended samples at 30 days.

For ferrihydrite amended sample, the known and likely iron reducers' abundance increased from 33.4% to 59.4% and 5.6% to 21.8% simultaneously. Both known iron reducers



Chlorobi group bacterium clone D25\_44 ( $\beta$ -Proteobacteria) and uncultured Acidobacteriaceae bacterium clone D12\_13 (Firmicutes) were present at initial and final time points of the experiment (Fig. 11). The abundance of clone 25\_44 ( $\beta$ -Proteobacteria) and clone D12\_13 (Firmicutes) changed from 19.4% to 16.2% and 5.6% to 10.8%. Additional iron reducers were found at the end of experiment that indicates that iron amendment can change microbial diversity of the system and can open new metabolic niche of iron reducers. New iron reducers microbes accounted for 32.4% of the total biomass which is significantly higher than other iron amendment samples. The known iron reducers are uncultured delta proteobacterium clone EtOH+Fe-c48 (uncultured  $\delta$ -proteobacteria), uncultured Desulfobulbaceae bacterium clone HT06Ba03 (uncultured  $\delta$ -proteobacteria) and Alpha proteobacterium pACH81 (uncultured  $\alpha$ -Proteobacteria). Thus, the diversity of other microorganisms together with known and likely iron reducers microorganisms changed noticeably during the experimental period which was confirmed by Shannon index and Simpson's index of diversity ( $H_{T=0} = 1.604$ ,  $H_{T=30} = 2.326$ ,  $D_{T=0} = 0.744$ ,  $D_{T=30} = 0.907$ ) (Table 04).

For lepidocrocite amended sample, the known and likely iron reducers' biomass increased from 26.3% to 41.7% and 0% to 22.2% simultaneously. Both known iron reducers Chlorobi group bacterium clone D25\_44 ( $\beta$ -Proteobacteria) and uncultured Acidobacteriaceae bacterium clone D12\_13 (Firmicutes) were present at initial and final time points of the experiment (Fig. 12). Clone 25\_44 ( $\beta$ -Proteobacteria) and clone D12\_13 (Firmicutes) changed from 15.6% to 27.7% and 5.4% to 8.4%. The known iron reducers were produced at the end time point is uncultured delta proteobacterium clone EtOH+Fe-c48 (uncultured  $\delta$ -proteobacteria). The diversity of other microorganisms together with known and likely iron reducers microorganisms changed significantly during the experimental period which was confirmed by Shannon index

and Simpson's index of diversity ( $H_{T=0} = 1.421$ ,  $H_{T=30} = 2.161$ ,  $D_{T=0} = 0.657$ ,  $D_{T=30} = 0.871$ ) (Table 04).

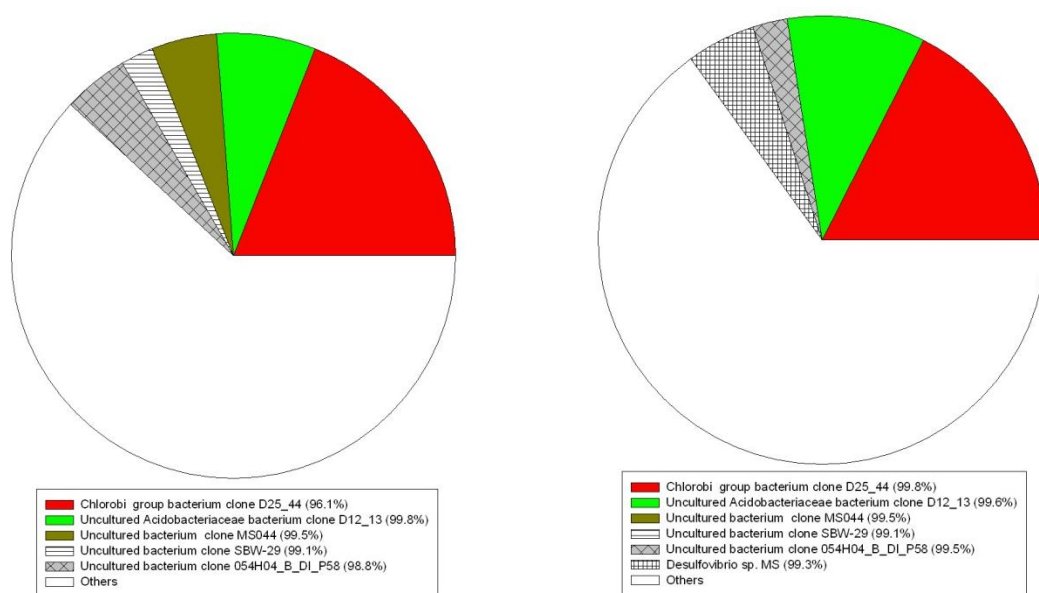
For  $Fe^{3+}$ EDTA amended sample, the known and likely iron reducers' biomass increased from 10.8% to 22% and 5.4% to 39.1% simultaneously. The increase was not as significant as ferrihydrite and lepidocrocite amended sample. Known iron reducers Chlorobi group bacterium clone D25\_44 ( $\beta$ -Proteobacteria) and uncultured Desulfobulbaceae bacterium clone HT06Ba03 (uncultured  $\delta$ -proteobacteria) were present at initial and final time points of the experiment. Clone 25\_44 ( $\beta$ -Proteobacteria) and clone clone HT06Ba03 (uncultured  $\delta$ -proteobacteria) changed from 15.6% to 27.7% and 5.4% to 8.4% (Fig. 13). No new known iron reducer microorganism was identified. The diversity of other microorganisms together with known and likely iron reducers microorganisms changed noticeably during the experimental period which was confirmed by Shannon index and Simpson's index of diversity ( $H_{T=0} = 1.831$ ,  $H_{T=30} = 2.672$ ,  $D_{T=0} = 0.743$ ,  $D_{T=30} = 0.924$ ) (Table 04).

Jaccard similarity coefficients were calculated comparing the microbial diversity with iron amended and un-amended sample at initial and final time points. Jaccard coefficients confirm that microbial diversity became more diverse at the end time points due to iron amendment. The changes are the following: i) ferrihydrite amended sample from 0.28 to 0.142, ii) lepidocrocite amended sample from 0.21 to 0.068 and iii)  $Fe^{3+}$ EDTA sample from 0.16 to 0.11.

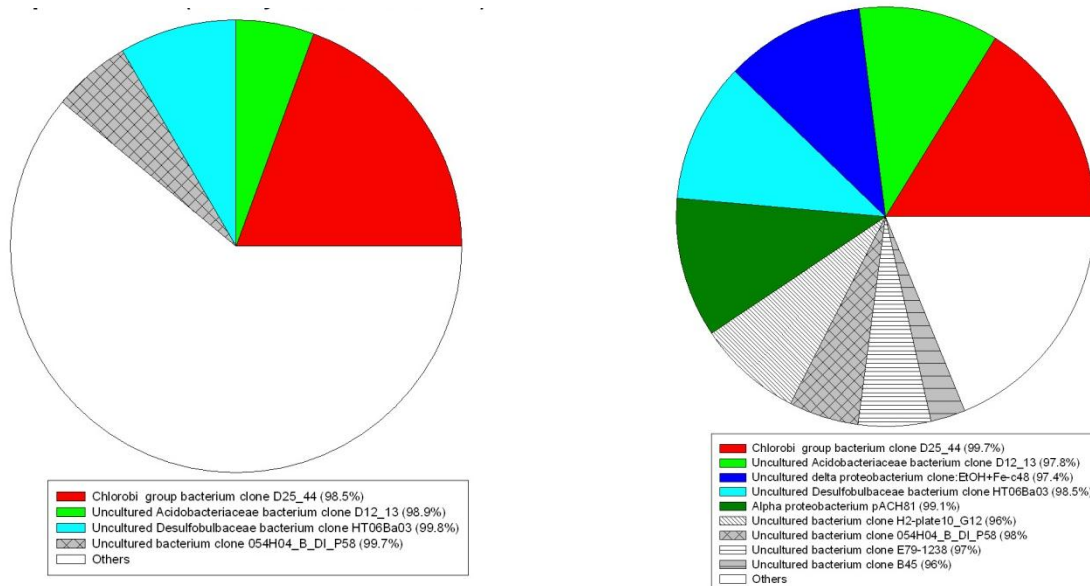
**Table 4:** Indices of microbial community diversity for iron amended incubations based on 16S rRNA gene clone libraries

sample	H <sup>a</sup>	D <sup>b</sup>	J <sup>d</sup>
(1) No iron amendment (T=0)	2.208	0.897	
(2) No iron amendment (T=30)	2.399	0.9025	
(3) Ferrihydrite amended (T=0)	1.604	0.744	0.28 (compare with 1)
(4) Ferrihydrite amended (T=30)	2.326	0.907	0.142 (compare with 2)
(5) Lepidocrocite amended (T=0)	1.421	0.657	0.21 (compare with 1)
(6) Lepidocrocite amended (T=30)	2.161	0.871	0.068 (compare with 2)
(7) Iron-EDTA amended (T=0)	1.831	0.743	0.16 (compare with 1)
(8) Iron-EDTA amended (T=30)	2.672	0.924	0.11 (compare with 2)

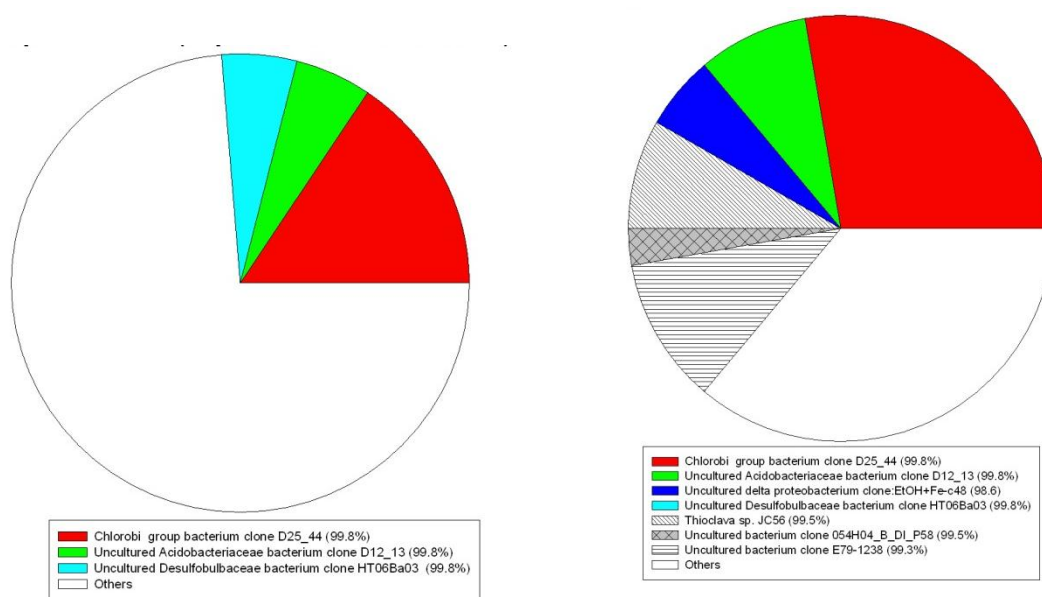
<sup>a</sup>Shannon index. <sup>b</sup>Simpson's index of diversity. <sup>c</sup>Jaccard similarity coefficient comparing the clone libraries of iron amended and unamended incubations at initial and final time points



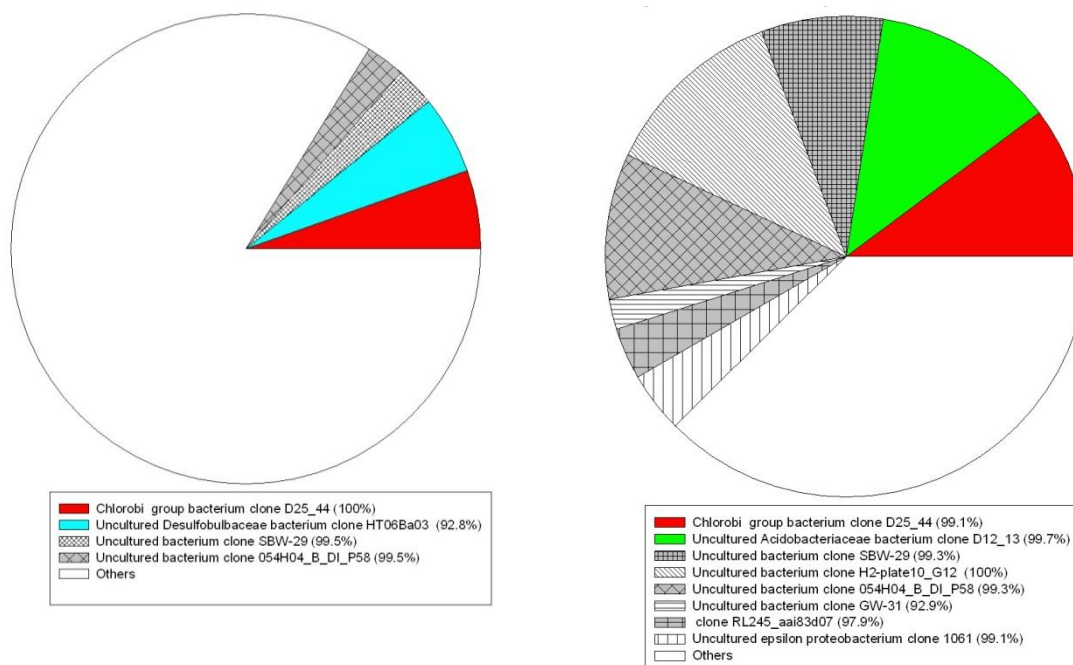
**Figure 10 :** Percent distribution of known and likely Fe<sup>3+</sup> reducers at time = 0 and time = 30 days for samples **not amended with Fe<sup>3+</sup>**. “Others” includes all organisms that cannot be categorized as known or likely Fe<sup>3+</sup> reducers. “Known” Fe<sup>3+</sup> reducers are identified in color sections. “Likely” Fe<sup>3+</sup> reducers are designated in the hatched/marked sections without color. “Others” are defined in the supporting information.



**Figure 11:** Percent distribution of known and likely  $\text{Fe}^{3+}$  reducers at time = 0 and time = 30 days for samples **amended with ferrihydrite**. “Others” includes all organisms that cannot be categorized as known or likely  $\text{Fe}^{3+}$  reducers. “Known”  $\text{Fe}^{3+}$  reducers are identified in color sections. “Likely”  $\text{Fe}^{3+}$  reducers are designated in the hatched/marked sections without color. “Others” are defined in the supporting information.



**Figure 12:** Percent distribution of known and likely  $\text{Fe}^{3+}$  reducers at time = 0 and time = 30 days for samples **amended with lepidocrocite**. “Others” includes all organisms that cannot be categorized as known or likely  $\text{Fe}^{3+}$  reducers. “Known”  $\text{Fe}^{3+}$  reducers are identified in color sections. “Likely”  $\text{Fe}^{3+}$  reducers are designated in the hatched/marked sections without color. “Others” are defined in the supporting information.



**Figure 13:** Percent distribution of known and likely  $\text{Fe}^{3+}$  reducers at time = 0 and time = 30 days for samples **amended with FeEDTA**. “Others” includes all organisms that cannot be categorized as known or likely  $\text{Fe}^{3+}$  reducers. “Known”  $\text{Fe}^{3+}$  reducers are identified in color sections. “Likely”  $\text{Fe}^{3+}$  reducers are designated in the hatched/marked sections without color. “Others” are defined in the supporting information.

### 3.5 Discussion

Iron (III) amendments increased the rate and extent of mineralization to  $\text{CO}_2$  over both the short and long term, and decreased the total mass of  $\text{CH}_4$  generated. Application of this approach would benefit wastewater treatment because the amount of carbon oxidized in standard septic systems is relatively small compared to municipal wastewater treatment, and sludge treatment/disposal from septic systems is costly and inefficient. Ferric iron is benign in terms of human or environmental health (at the concentrations used in this study), and if managed properly it can be added to septic systems without disrupting flow or harming infrastructure. Ferric iron escaped to downstream treatment units will be precipitated inside the sand filtration/soil treatment units and can be effective as electron acceptor and removal of pathogens.

Short term and long term carbon mineralization are both critical in septic systems. Carbon enters these systems primarily in the aqueous phase or as suspended solids (Crites and Tchobanoglous, 1998; Baumann et al, 1977) and the aqueous phase moves through the system in a matter of days, whereas the “sludge layer” remains for weeks and even months or possibly years (Crites and Tchobanoglous, 1998, Baumann et al, 1977). The short term mineralization data presented above demonstrate that ferric iron will increase the extent of mineralization within 30 hours of amendment, which will influence carbon that exists primarily in the aqueous phase as it moves through a septic tank. The long term mineralization data indicate that carbon that remains in the septic tank will be: a) mineralized to a greater extent when  $\text{Fe}^{3+}$  is present, and b) will be preferentially mineralized to  $\text{CO}_2$  rather than  $\text{CH}_4$  when  $\text{Fe}^{3+}$  is present. Although both  $\text{CO}_2$  and  $\text{CH}_4$  are greenhouse gases, methane is significantly more potent and has garnered recent attention from the Water Environment Research Foundation (WERF), which commissioned a

study to understand the contribution of septic systems to methane production (RFP, WERF, 2009).

The amendments were selected to encompass key carbon molecules that are relevant wastes: low molecular mass organic acids (acetate, butyrate, lactate, and propionate), carbohydrate monomers (glucose) and polymers (starch), and lipids (oleic acid). Septic systems also receive large cellulose inputs (e.g. toilet paper), but anoxic cellulose biodegradation is very limited and this fraction typically ends up as a true sludge layer (Cooney et al., 1999; Benner et al., 1984)

Although septic tanks are efficient for a minimally engineered system, they leave a large residual carbon pool available in the sludge layer (which must be pumped out and treated further) and in the aqueous or suspended solids fraction, which flows into a leach field or directly discharges into the natural environment (Crites and Tchobanoglous, 1998). The fraction of carbon that exits the tank in the water (either dissolved or suspended) enters the leach field and eventually site soil. In an ideal application soil microorganisms will further oxidize the carbon and remove the BOD potential before it enters surface or ground water and can contribute to eutrophication and general water body degradation (Charles et al., 2005; Taebi and Droste, 2004). However, the native soil microorganisms may not be capable of oxidizing the carbon flowing out of the septic tank at rates that would prevent it from entering natural water bodies (Safferman et al., 2004)

In addition, the potential for methane in native environments under anoxic conditions, and not just the septic tank itself, is also problematic. It is more reasonable to engineer the septic tank to maximize carbon oxidation than to rely on processes that may or may not occur once the carbon exits the system. Though suppression of methanogenesis due to ferric iron amendment is

found in other anaerobic sedimentary environment, it is the first study that proves the effectiveness of ferric iron amendment in such a complex and diverse septic environment.

All forms of  $\text{Fe}^{3+}$  increased carbon mineralization for at least one carbon substrate. Ferrihydrite and lepidocrocite are the two semi-solid iron phases added to the experiments. As would be expected these forms of  $\text{Fe}^{3+}$  immediately sink and are associated with the sludge layer on the bottom of the experimental bottle. The remaining  $\text{Fe}^{3+}$  phases were all soluble (chelated) forms and can mix thoroughly throughout the water and sludge layers.

Three different forms of iron were used in this study: soluble (ferric citrate, ferric phosphate), poorly crystalline (ferrihydrite and lepidocrocite) and chelated forms (ferric NTA and ferric EDTA). Ferric citrate and ferric phosphate consistently did not stimulate higher carbon oxidation compared to unamended systems. Ferric citrate can be toxic to some  $\text{Fe}^{3+}$  reducing microorganisms, and if the citrate is fermented faster than the  $\text{Fe}^{3+}$  is reduced the precipitated  $\text{Fe}^{3+}$  may not be bioavailable (Lovley, 2006). However, specific  $\text{Fe}^{3+}$  forms are reduced by specific microbial communities (Lovley, 2006); thus it is possible that the appropriate organisms were not present in the septic samples to reduce  $\text{Fe}^{3+}$  citrate or  $\text{Fe}^{3+}$  phosphate. However, both ferric citrate and ferric phosphate suppressed methane production (Table 3). While these  $\text{Fe}^{3+}$  forms did not increase mineralization to  $\text{CO}_2$  significantly in the short or long term, they did inhibit methanogenesis. Redox potential of iron reducing environment is unsuitable for methanogenesis. This demonstrates that only low  $\text{Fe}^{3+}$  concentrations are necessary to shift metabolism away from methane production, which will be useful if methane suppression is the only goal.



Poorly crystalline  $\text{Fe}^{3+}$  oxides (ferrihydrite) serve as the electron acceptor for complete carbon oxidation (to  $\text{CO}_2$ ) in subsurface environments (Lovley and Philips, 1987b; Lovley, 2006). It is the most “environmentally relevant” form of  $\text{Fe}^{3+}$  with respect to microbial respiration, but other  $\text{Fe}^{3+}$  forms can stimulate carbon oxidation in natural and engineered systems. Lepidocrocite is a more crystalline  $\text{Fe}^{3+}$  solid (Cornell et al., 2003; Hansel et al., 2005). It is slightly less bioavailable than ferrihydrite, but is a naturally occurring mineral and therefore  $\text{Fe}^{3+}$  reducers will encounter it in situ.

Chelated forms of  $\text{Fe}^{3+}$  such as ferric EDTA and NTA are synthetic, bioavailable and soluble forms of  $\text{Fe}^{3+}$  (unlike solid  $\text{Fe}^{3+}$  forms, which precipitate). EDTA and NTA are very resistant to anaerobic degradation (Yuan and VanBriesen, 2008) and therefore will remain as ligands for  $\text{Fe}^{3+}$  in strictly anoxic systems, which accelerates  $\text{Fe}^{3+}$  reduction (Lovley, 2006). Rates of bacterial  $\text{Fe}^{3+}$  reduction in the presence of dissolved chelating agents correlate with the thermodynamic stability constants of the  $\text{Fe}^{3+}$ -organic complexes, implying that chemical speciation governs  $\text{Fe}^{3+}$  bioavailability (Haas and Dichristina, 2002).

Ferric NTA and EDTA inhibited short term mineralization of lactate, but increased long term mineralization. Chelating ligands like EDTA and NTA have the potential to bind the iron so tightly that  $\text{Fe}^{3+}$  is not readily bioavailable to all  $\text{Fe}^{3+}$  reducers (Zhu et al, 2009). Additionally, lactate may need fermentative conversion to acetate and  $\text{H}_2$  before  $\text{Fe}^{3+}$  reducers can transform them to  $\text{CO}_2$ . Thus it took longer acclimation periods for some  $\text{Fe}^{3+}$  reducing cells, in this case lactate-oxidizing organism in our study.

Once acclimated, however, ferric NTA/EDTA can stimulate  $\text{Fe}^{3+}$  reduction because the soluble  $\text{Fe}^{3+}$  is much more bioavailable than insoluble forms such as ferrihydrite. Moreover,

variability in size, charge and structure of chelated forms of  $\text{Fe}^{3+}$  compared to mineral oxides (ferrihydrite, lepidocrocite) may allow them to gain access to enzyme active sites of cells that may not be accessible to insoluble mineral oxides (Ruebush et al., 2006). Chelated  $\text{Fe}^{3+}$  has been demonstrated to stimulate oxidation of many natural organic compounds such as propionate and lactate, as well as recalcitrant organic compounds such as benzene and other petroleum hydrocarbons (Coates et al., 1998; Coates et al., 1995; Lovley et al., 1994)

The solubilization effect of  $\text{Fe}^{3+}$  is more significant with EDTA than NTA because EDTA is a stronger  $\text{Fe}^{3+}$  ligand ( $\log K_{\text{Fe(III)NTA}}=17.83$ ,  $\log K_{\text{Fe(III)EDTA}}=27.57$ ) (Urrutia et al, 1998). Here ferric EDTA mineralized butyrate and starch to a higher extent than ferric NTA; though NTA performed better than EDTA for other carbon compounds. The results indicate that multiple factors (e.g. toxicity, acclimation period, microbial community composition) are involved in the  $\text{Fe}^{3+}$  reduction rate and extent differences than just solubility issues. The chelated forms of  $\text{Fe}^{3+}$  increased oxidation for most carbon molecules more so than the poorly crystalline form - ferrihydrite.

Acetate is the primary carbon intermediate in the breakdown of all organic matter (Lovley and Phillips, 1987b; McCarty and Smith, 1980) and it is one precursor for methane. Shifting acetate metabolism towards  $\text{CO}_2$  production limits the amount of acetate available to become methane. Coupled with a  $\text{Fe}^{3+}$  reducer dominated microbial community, there is limited potential for acetoclastic methanogenesis. The alternate pathway leading to methane is via hydrogen and  $\text{CO}_2$ . While  $\text{CO}_2$  ( $\text{HCO}_3^-$ ) is abundant in the system, hydrogen is very limited when  $\text{Fe}^{3+}$  reduction is the dominant terminal electron accepting process (less than 0.1nM) (Chapelle and Lovley, 1992).  $\text{Fe}^{3+}$  reducing microorganisms metabolize molecular  $\text{H}_2$  to a

steady-state level that is too low to sustain methanogenesis. Methanogens require 5nM  $H_2$  or greater in solution, which is not present when  $Fe^{3+}$  reduction is stimulated.

Methane inhibition was due to a combination of effects mediated by  $Fe^{3+}$  reducers: first, the total microbial community shifted to enrich  $Fe^{3+}$  reducers and limit methanogens (which is shown using 16S rRNA bases quantitative molecular analyses), second, acetate was metabolized more readily by  $Fe^{3+}$  reducers than methanogens – leaving little or no acetate to generate methane, and finally, the steady-state dissolved  $H_2$  was too low to favor methanogens. Only in the unamended systems was methane production dominant after a long incubation period.  $Fe^{3+}$  amendment(s) are an effective method to limit methane in septic systems, and this strategy may be required as greenhouse gas emissions in on-site wastewater are scrutinized more closely. The few cases in which  $Fe^{3+}$  amended systems generated methane (lepidocrocite amendment for butyrate and lipid; Table 3) were exceptions, and were likely due to mixed microbial communities being present in the bottles. Although this is not typical, methanogens and  $Fe^{3+}$  reducers can coexist under certain conditions. In both cases, however, the extent of mineralization to  $CO_2$  was much greater in the presence of  $Fe^{3+}$ , and methane production was greater when  $Fe^{3+}$  was absent (Table 3).

Iron reduction opened new metabolic niche in onsite wastewater treatment systems. Three selected iron reduction dominated samples (ferrihydrite, lepidocrocite and ferric EDTA) were collected from wastewater together with un-amended sample. ARDRA analysis shows there is a significant increase of known iron reducers due the iron amendment ranging from 11.2% to 26%. The known iron reducers did not increase in unamended sample rather decreased by 3.5%. There was also likely iron reducers increased in iron amended samples. Likely iron

reducers are known anaerobic bacteria with potential of reducing iron and were significantly increased in number due to iron amendment.

The known iron reducers found in this study are Chlorobi group bacterium clone D25\_44 ( $\beta$ -Proteobacteria), uncultured Acidobacteriaceae bacterium clone D12\_13 (Firmucutes), uncultured bacterium clone MS044 (Firmucutes), uncultured delta proteobacterium clone EtOH+Fe-c48 (uncultured  $\delta$ -proteobacteria), uncultured Desulfobulbaceae bacterium clone HT06Ba03 (uncultured  $\delta$ -proteobacteria) and Alpha proteobacterium pACH81 (uncultured  $\alpha$ -Proteobacteria). Those microorganisms are closely associated with the following microorganisms that confirm that they are related to iron reduction: iron-reducing enrichment clone Cl-A4 (Wang et al, 2009; Chouari et al., 2010), *Geothrix fermentans* strain H5 (Lonergan et al., 1996; Liesack et al., 1994), iron-reducing bacterium enrichment culture clone HN117 (Wang et al, 2009), *Geobacter metallireducens* (Akob et al., 2008; Lovley et al., 1993), uncultured  $\delta$ -proteobacteria (Kunapuli et al., 2007) and uncultured  $\alpha$ -Proteobacteria (Kim and Rhee, unpublished).

Several dissimilatory iron-reducing bacteria (DIRB) have been isolated from a variety of anoxic environments includes *Geobacter*, *Shewanella*, *Pelobacter*, *Geovibrio*, *Geospirillum*, *Ferrimonas*, *Geothrix*, *Desulfuromusa*, and *Desulfuromonas*, *Bacillus infernus*, *Thermoterrabacterium*, *Deferribacter thermophilus*, and *Thermoanaerobacter* spp. Also, there are several reports of enrichment cultures of thermophilic bacteria that are capable of dissimilatory iron reduction (references cited in Kieft et al., 1999). It is also known that some sulfate reducing bacteria are also able to reduce  $\text{Fe}^{3+}$  (Taylor 1998). Dissimilatory  $\text{Fe}^{3+}$ -reducing bacteria such as *Shewanella*, *Geobacter* etc capture energy for anaerobic growth via oxidation of organic matter coupled to enzymatically catalyzed dissimilatory reduction of  $\text{Fe}^{3+}$  as terminal electron acceptor (TEA) (Haas and Dichristina, 2002). Several of the microorganisms found are

unique and different than commonly found iron reducers described above. The data suggests that several of the microorganisms found in this study can be a potential candidate for dominant iron reducer capable of oxidizing complex carbon molecules in onsite wastewater systems.

## CHAPTER 4 FERRIC IRON AMENDMENT INCREASES PHOSPHORUS REMOVAL AS VIVIANITE IN ON-SITE WASTEWATER (SEPTIC SYSTEMS)

### 4.1 Abstract

Onsite wastewater treatment systems (OWSs) serve approximately 25% of the U.S. population. Phosphorus, a major nutrient that causes eutrophication, accumulates in onsite wastewater system from human and domestic wastes. Phosphate is of particular interest because its growth limiting effects on microbes in natural aqueous environments. Phosphorus removal from wastewater can be achieved either through chemical removal, advanced biological treatment or a combination of both such as calcium, iron, aluminium salts, enhanced biological phosphorous removal (EBPR) etc. We proposed a novel technique to apply in anaerobic onsite wastewater by leveraging iron reduction to oxidize more carbon and precipitate phosphorus with reduced iron as vivianite. Biological  $\text{Fe}^{3+}$  reduction can lead to precipitation of the completely insoluble mineral vivianite  $\text{Fe}_3(\text{PO}_4)_2 \cdot (\text{H}_2\text{O})_8$ , which would remain insoluble in secondary treatment sludge. It can be stimulated by adding bioavailable  $\text{Fe}^{3+}$  to promote  $\text{Fe}^{3+}$  reduction. Carbon-14 radiolabeled acetate, xylose, starch, and oleic acid were used to demonstrate that short and long term carbon oxidation is increased when different forms of  $\text{Fe}^{3+}$  (ferrihydrite, lepidocrocite,  $\text{Fe}^{3+}$ NTA and  $\text{Fe}^{3+}$ EDTA) are amended to septic wastewater. The rates of carbon mineralization to  $^{14}\text{CO}_2$  increased 2 to 3 times (relative to unamended systems) in the presence of  $\text{Fe}^{3+}$ . Pure culture growth experiments in ferric citrate media with *Geobacter metallireducens* clearly showed extensive iron reduction in presence of donor and subsequent phosphate removal (>90%). Both TEM-EDAX and XRD technique confirms the precipitant as vivianite when additional  $\text{Fe}^{2+}$  present in wastewater. Diluted WW samples showed increased COD removal with complete removal of phosphate for some iron amended samples compare to un-amended

samples. Similar removal characteristics were observed for diluted samples with phosphate added initially. Ferric citrate, ferrihydrite and ferrihydrite+AQDS completely removed (>95.5% or higher) phosphate in the original wastewater sample. Ferric citrate and ferrihydrite+AQDS performed better (>97.5 or higher) when extreme phosphate loading condition was evaluated. Lepidocrocite, ferric NTA and Ferric EDTA also removed phosphate from experimental samples. The result clearly shows that iron reduction coupled with phosphate removal can be an effective strategy for onsite wastewater systems.

## 4.2 Introduction

Onsite wastewater treatment systems (OWS), also referred to as septic systems are dominant in developing countries. Moreover, one-fourth of US population depends on decentralized wastewater treatment (Conn et al, 2006, Lowe and Siegrist, 2008). Phosphorus, a major nutrient that causes eutrophication, accumulates in onsite wastewater system from human and domestic wastes (Alexander and Stevens, 1976; Barnes et al., 1984). Domestic wastewater is considered one of the major sources of phosphates and nitrogen compounds to the natural environment (Svanks, 1971). Phosphate is of particular interest because its growth limiting effects on microbes in natural aqueous environments (Schindler, 1977; Hudson et al., 2000; Wu et al., 2000). Approximate concentrations of phosphorus (5 to 20 mg/L) found in sewage effluent exceed the lower requirement ([approximately] 0.03 mg/L) for algal growth ([approximately] 0.03 mg/L) (Dillon and Rigler 1974; Schindler 1977).

The need to control phosphorus loading to receiving waters and prevent eutrophication has been well documented (Vollenweider, 1985). Eutrophication has many negative effects on aquatic ecosystems such as proliferation of algae, degradation of water quality, oxygen shortages, loss of natural habitats, negative impacts on aquaculture and shellfisheries etc (Carpenter et al., 1998). Onsite wastewater usually contains different forms of phosphorus such as orthophosphate, organic phosphorus, condensed phosphates (pyrophosphate or tripolyphosphate). Organic phosphorus and condensed phosphates can be hydrolyzed completely to orthophosphate at certain conditions (Finstien and Hunter, 1967; De Jong, 1985).

Phosphorus removal from wastewater can be achieved either through chemical removal, advanced biological treatment or a combination of both (Wang, et al., 2006). The chemical



phosphorus removal techniques involve the addition of calcium, iron and aluminium salts to achieve phosphorus precipitation by various mechanisms (Yeoman et al, 1988). Generally, aluminium sulphate (alum) is considered the best precipitant (Lin & Carlson, 1975), followed by  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{Fe}_2(\text{SO}_4)_3$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{Ca}(\text{OH})_2$  (Metcalf and Eddy, 1993).

Phosphorus mineral precipitation is practically acceptable because it immobilizes phosphorus as solid compounds without the need for excessive biomass or additional oxygenation. Thus, overall sludge load of the system can be reduced, which is critical to efficient sludge treatment (Wang et al, 2005) and unit operations. In addition, these minerals are more stable than biologically accumulated sludge (Wang et al, 2005). To date chemical precipitation has been suggested or reported (Santruckova et al, 2004; Sondergaard et al, 2003; Wang et al, 2005) but biological precipitation is possible. The proposed work will focus on biological phosphorus removal via secondary reactions catalyzed by  $\text{Fe}^{3+}$  reduction, namely vivianite production ( $\text{Fe}_3(\text{PO}_4)_2 \cdot (\text{H}_2\text{O})_8$ ).

Vivianite is an important phosphate mineral in many natural and environmental aquatic systems (Borno and Tomson, 1994). Vivianite has been identified in a number of environments including river sediments (up to 20% by mass) (Hearn et al., 1983; Woodruff et al., 1999), canals (Dodd et al., 2000), lakes (Nriagu and Dell, 1974; Nembrini et al., 1983), wastewater sludges (Frossard et al., 1997) and river delta muds (Bailey et al., 1998) although not in all environments where the solubility of vivianite is exceeded (Hearn et al., 1983).

Vivianite has been shown to be stable under reducing conditions and high  $\text{Fe}^{2+}$  and  $\text{PO}_4^{2-}$  activities, and low sulphide activities (Rosenquist, 1970; Nriagu, 1972; Emerson and Widmer, 1978). In phosphate-rich environments, vivianite ( $\text{Fe}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}$ ) is an important sink for

dissolved  $\text{Fe}^{2+}$  and is considered as a very stable mineral due to its low solubility at neutral pH (Miot et al, 2009). Further increases in iron concentrations in solutions already at equilibrium with vivianite (i.e., the effluent) would be expected to cause additional precipitation of vivianite. Theoretical calculation showed vivianite precipitation is plausible in septic system (Robertson et al, 1998).

Microorganisms play an important role in the natural environment by determining the speciation of Fe (Lemos et al, 2007). Microbial communities affect the pH and redox of natural waters, determine the form of the iron in solution, as well as the iron compounds that are precipitated (Brown et al, 1999). Dissimilatory iron reducing bacteria (DIRB) mediate the formation of Fe mineral phases by delivering  $\text{Fe}^{2+}$  to the bulk solution after attachment to and reduction of  $\text{Fe}^{3+}$  minerals at the cell surface (Arnold et al., 1986b; Lower et al., 2001), away from the cell via electron shuttling mechanisms (Lovley et al., 1996; Newman and Kolter, 2000), or possibly via soluble cytochromes (Seeliger et al., 1998). Many microorganisms, e.g., iron-reducing, sulfate-reducing, and some fermenting bacteria, are capable of using humics (HS) as an electron acceptor for anaerobic oxidation of organic and inorganic electron donors (Lovley et al, 1996; Coates et al., 1998; Benz et al., 1998; Cervantes et al., 2002).

$\text{Fe}^{3+}$  reducing microorganisms are ubiquitous, including septic sludge; however, in these systems they are limited by low (or absent) bioavailable  $\text{Fe}^{3+}$  (Castillo-Gonzalez and Bruns, 2005; Coates et al, 2005; Ivanov et al., 2004; Kim et al, 2004). Biological  $\text{Fe}^{3+}$  reduction can lead to precipitation of the completely insoluble mineral vivianite  $\text{Fe}_3(\text{PO}_4)_2 \cdot (\text{H}_2\text{O})_8$ , which would remain insoluble in secondary treatment sludge. It can be stimulated by adding bioavailable  $\text{Fe}^{3+}$  to promote  $\text{Fe}^{3+}$  reduction. Vivianite appeared to be the stable end product and the mean coherence length was influenced by the rate of  $\text{Fe}^{3+}$  reduction (Glasauer S. et al, 2003).

Phosphate concentration dependent vivianite formation is favored at circumneutral pH or above. This is a simple amendment that can easily be “retrofitted” to any standard on-site wastewater (septic) system. Moreover,  $\text{Fe}^{3+}$ -reducing microorganisms can utilize a wide variety of substrates including carbohydrates, lipids, aromatic and aliphatic compounds, amino acids and its daughter products that can reduce COD at a significantly shorter time. Microbial  $\text{Fe}^{3+}$  reduction is an energetically favorable process, and in the natural environment,  $\text{Fe}^{3+}$ -reducing bacteria can outcompete and inhibit both sulfate-reducing and methanogenic bacteria (references cited in Coates et al., 2005). Thus,  $\text{Fe}^{3+}$  reduction can significantly reduce sulfide production and thus can promote phosphate removal as vivianite.

The objectives of the present study were to characterize the effect of iron reduction on phosphate removal together with its effects on carbon oxidation and gas production. The results demonstrated that carbon oxidation to carbon-dioxide was enhanced by iron reduction. Both solid and soluble forms of iron were capable of phosphate removal at greater extent and at a short period compare to unamended system. Growth experiment with subsequent TEM and XRD studies confirms that vivianite is the most likely candidate for removal of phosphate from the system when  $\text{Fe}^{2+}$  is present.

## 4.3 Materials and Methods

**4.3.1 Characteristics:** Septic wastewater was collected from a single family household in St Joseph, Champaign (County), Illinois. A “sludge judge” bailer (a long, hollow, clear plastic pole with a bottom-check-valve marked in 1-foot increments) was used to collect representative sample from flocculated and settled volume of septic sludge. Septic wastewater was sealed without a headspace with airtight closures, packed in ice chests and immediately transported to Newmark Civil Engineering Laboratory, Urbana, IL. Initial characterization of the septic water was conducted for pH, filtered and total COD,  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ ,  $\text{PO}_4^{3-}$ ,  $\text{NO}_3^-$  and  $\text{SO}_4^{2-}$  (Greenberg et al, 1992). Samples were stored at 4°C for further experiment and analysis.

**4.3.2 Growth experiments:** Cells (*Geobacter metallireducens*: GS-15) were grown anaerobically in ferric citrate media with an electron donor (i.e., 20 mM acetate) and an electron acceptor (i.e., 50 mM Fe(III) citrate). Anaerobic ferric citrate media was prepared by sparging the medium bottle with  $\text{H}_2:\text{CO}_2$  (80:20, vol/vol). Cells (10%) at logarithmic growth condition were transferred to new ferric citrate media. Three separate experimental (with triplicates) GS-15 growth conditions in ferric citrate media were investigated with proper control. The conditions of the four batches of incubation were following: electron donor acetate amended ferric citrate media with i) GS-15 with 10 mM  $\text{PO}_4^{3-}$  added initially ii) GS-15 with 10 mM  $\text{PO}_4^{3-}$  added at 30 hrs. iii) GS-15 with no  $\text{PO}_4^{3-}$  addition and iv) No GS-15 with 10 mM  $\text{PO}_4^{3-}$  addition. Acetate (20mM) was amended as the sole electron donor. Cells were incubated at 30°C. Samples were collected periodically via anaerobic syringe and needle.  $\text{Fe}^{2+}$  accumulation and soluble phosphate concentration was measured in all experimental tubes.

**4.3.3 Transmission electron microscopy (TEM):** Transmission electron microscopy was used to characterize the mineral products derived from the reduction of ferric citrate in presence of *Geobacter metallireducens*. Negative staining technique was used for transmission electron microscopy (TEM). Growth medium with cells and precipitates were centrifuged at 3000 rpm for 1 min. After initial centrifugation, the supernatant was discarded and hepes buffer was used for subsequent centrifugation and disposal of supernatant to clean the sample. The centrifugation was repeated 4 times before adding fixative 2% ammonium molybdate to the centrifuged pellets and was stored at 4° C before placing it on a carbon grid.

The samples were then placed as a drop on parafilm and a copper grid, coated with both formvar plastic and carbon, was placed on top of the specimen droplet for 5-15 minutes depending upon the concentration. The excess sample was then removed with filter paper and the grid placed on 2% ammonium molybdate for 2 minutes. The grid was then dried by removing the excess fluid with filter paper, placed into a grid box and covered with drierite crystal. The grid was then examined in the transmission electron microscope-energy-dispersive X-ray (TEM-EDS) system and observed at 20,000x or higher magnification.

**4.3.4. X-ray diffraction (XRD):** Solid sample was filtered anaerobically both from growth and septic experiment for subsequent XRD analysis. The whole sample preparation procedure was carried out in an anoxic glove-box to ensure strict anoxic conditions. Solid samples were collected using a filter cartridge in a Whatman filter paper. The samples were dried inside the glove-box and were inserted into glass capillary sealed later at both sides to ensure anaerobic condition during analysis.

Powder XRD data were collected on a Bruker General Area Detector Diffraction System (GADDS) equipped with a four-circle diffractometer and HiStar multiwire area detector. A Bruker M18XHF<sup>22</sup> rotating anode generator operating at 50kV and 40mA supplied the graphite monochromated incident beam. The sample was loaded without modification into a 0.4mm special glass capillary and exposed for 2 hours for each of two frames. Scatter from the glass capillary was removed by coincidence correction before merging the two frames for integration. Unless otherwise noted, the final spectrum was truncated to remove boundary anomalies and normalized to facilitate pattern matching.

**4.3.5. Diluted wastewater experiment:** Onsite wastewater samples were diluted 25 times to understand the change of COD due to iron amendment distinctly. Two separate conditions were investigated in the diluted onsite wastewater. In-situ orthophosphate was significantly low in the experimental bottles due to the dilution. Two separate batches were run i) phosphate added after 20 days of incubation with additional 12 mM Fe<sup>3+</sup> (batch 01) and ii) phosphate added initially (batch 02). 4 different Fe<sup>3+</sup> types (20 mM) were used for the experiment with one ferrihydrite + AQDS (5 mM) amended incubation. The iron types used are ferrihydrite, lepidocrocite, ferric EDTA and ferric NTA .

Serum bottles (125 mL) and anaerobic tubes (28 mL) were used for the two batches of experiment. 50 and 10 mL diluted anaerobic wastewater incubation were prepared by bubbling the solution for 30 mins and 6 mins with 90:10 (vol/vol) N<sub>2</sub>:CO<sub>2</sub> that had been passed over hot copper filings to remove traces of oxygen. Then the head space was flushed with 90:10 (vol/vol) N<sub>2</sub>:CO<sub>2</sub>. Iron and phosphate amendment to the experimental bottles were done using anaerobic method described elsewhere (Wei and Finneran, 2011). Samples were collected periodically via anaerobic syringe and needle for subsequent analysis. Total COD, aqueous Fe<sup>2+</sup> and phosphate

were measured for batch 01 whereas the status of aqueous  $\text{Fe}^{2+}$  and phosphate were tracked for batch 02 incubation.

#### **4.3.6. Septic wastewater experiment with no dilution:**

##### **4.3.6a. Phosphate removal experiment.**

Onsite wastewater samples with no dilution were used to identify the characteristics of phosphate removal in original wastewater samples due to different iron amendment. Three different types of  $\text{Fe}^{3+}$  amendment (10 mM) with one additional ferrihydrite (10 mM) + AQDS (2 mM) amended anaerobic 10 mL samples were prepared by bubbling the solution for 6 mins with 90:10 (vol/vol)  $\text{N}_2:\text{CO}_2$  that had been passed over hot copper filings to remove traces of oxygen. For each type of  $\text{Fe}^{3+}$  amendment, two different types of conditions have been explored i) original wastewater sample with no additional phosphate amendment to simulate field conditions and ii) original wastewater sample with additional 62 mg/L phosphate to simulate extreme conditions of phosphorus loading (rain event, excessive domestic loading etc) in the onsite wastewater systems. The iron types used are ferric citrate, ferrihydrite, ferric NTA and ferrihydrite + AQDS.

**4.3.6b. Batch  $^{14}\text{C}$  oxidation experiments.** 10 mL triplicate anaerobic samples of onsite wastewater + 10 mM iron forms (three types with additional one with AQDS) + 0.1 mL  $^{14}\text{C}$  carbon compounds (4 types) were run in 28 mL anaerobic pressure tubes (Bellco Glass, Inc.) with appropriate controls to quantify mineralization potential (Lovley et al., 1995) of the carbon compounds. 9 mL anaerobic liquid (wastewater) samples were prepared by sparging the medium tube with  $\text{N}_2\text{-CO}_2$  (80:20 [vol/vol]) for 6 minutes and flushing the headspace for 1 min. All transfers and incubations were performed under the same strict anoxic conditions (Cozzarelli et

al, 2000). The tubes were sealed with thick butyl rubber stoppers and aluminum crimps (Bellco Glass, Inc.). All incubations were at 30 °C for 20 days.

10 mM concentration of ferrihydrite (FeGel), lepidocrocite,  $\text{Fe}^{3+}$  NTA and  $\text{Fe}^{3+}$  EDTA were added to incubation tubes from stock iron solution before amendment of radiolabeled carbon. 0.1  $\mu\text{Ci}$  or  $2.22 \times 10^5$  disintegrations per minute (dpm) of all individual [ $^{14}\text{C}$ ]-labeled carbon compounds were injected anoxically into the incubation tubes with a syringe and needle that had been flushed with  $\text{N}_2$ .  $^{14}\text{C}$  carbon compounds used were 2- $^{14}\text{C}$ -acetate (Moravek Biochemicals, Inc) , U- $^{14}\text{C}$ -xylose (Moravek Biochemicals, Inc), U- $^{14}\text{C}$ -starch (American Radiolabeled Chemicals, Inc) and 1- $^{14}\text{C}$ -oleic acid (lipid; Moravek Biochemicals, Inc).

1 mL headspace gas samples were collected using 1 mL gas-tight syringe that had been flushed with anoxic gas to track  $^{14}\text{CO}_2$  (dpm) and  $^{14}\text{CH}_4$  (dpm) by separating using a gas chromatograph (GC) equipped with a gas proportional counter (GPC) (Model GC Ram: IN/US Corporation, Tampa, FL). Total production of  $^{14}\text{C}$ -labeled inorganic carbon was calculated from the distribution of  $^{14}\text{CO}_2$  in the septic wastewater and the headspace, as determined by adding  $\text{H}^{14}\text{CO}_3^-$  standards to the septic samples using a scintillation counter. The proportion of  $\text{H}^{14}\text{CO}_3^-$  versus  $^{14}\text{CO}_2$  arising from  $\text{H}^{14}\text{CO}_3^-$  was quantified at each time point to calculate a pH dependent ‘partition’ value, which was used for measuring percent (%) mineralization of each of the  $^{14}\text{C}$  carbon compounds.



The mineralization equation is:

$$\text{Partition} = (Y_{\text{HCO}_3} * (V_{\text{head space}} / V_{\text{sample}}) / Z_{\text{HCO}_3})$$

$$\% \text{ of } [^{14}\text{C}]\text{-compounds recovered as } ^{14}\text{CO}_2 \text{ or } ^{14}\text{CH}_4$$

$$= (Y_{[^{14}\text{C}] \text{ compound}} \cdot V_{\text{head space}} / \text{partition}) / (Z_{[^{14}\text{C}] \text{ compound}})$$

here,  $Y_{\text{HCO}_3}$  = radioactivity of  $\text{CO}_2$  measured in the head space of  $\text{H}^{14}\text{CO}_3^-$  amended sample (dpm),  $Z_{\text{HCO}_3}$  = total radioactivity of  $\text{H}^{14}\text{CO}_3^-$  amended to the  $\text{H}^{14}\text{CO}_3^-$  amended sample (dpm),  $Y_{[^{14}\text{C}] \text{ compound}}$  = radioactivity of  $\text{CO}_2/\text{CH}_4$  measured in the head space of  $[^{14}\text{C}]$  - amended sample (dpm/mL),  $Z_{[^{14}\text{C}] \text{ compound}}$  = total radioactivity of  $[^{14}\text{C}]$  compound amended to the  $[^{14}\text{C}]$  - amended sample (dpm),  $V_{\text{head space}}$  = total volume of the head space of the anaerobic tube (mL),  $V_{\text{sample}}$  = volume of sample injected in GC-GPC for radioactivity measurement.  $^{14}\text{CH}_4$  does not require a partition value, as we assumed aqueous saturation and equilibrium within the headspace.

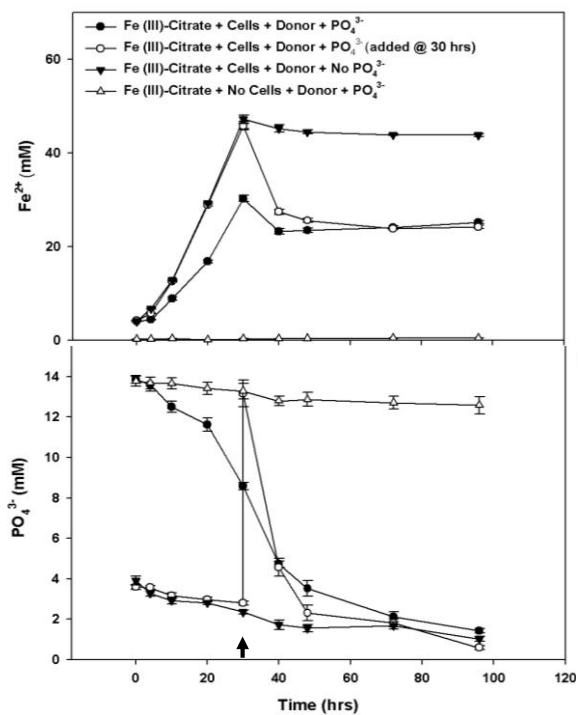
**4.3.7 MINEQL+ modeling:** MINEQL+ Ver 4.5 modeling was used to mimic the equilibrium  $\text{Fe}^{2+}$  and  $\text{PO}_4\text{-P}$  concentration for both growth and septic experiment. MINEQL+ Ver 4.5 determined the saturation indices of the solids that can precipitate in ferric citrate media (growth experiment) and septic sample (septic experiment). Saturation index (SI) is defined as  $\text{SI} = \text{Log}(Q/K_{\text{sp}})$ ; where  $Q$  = the ion product for the solid and  $K_{\text{sp}}$  = the solubility constant for the solid. Three types of values are important for solubility equilibrium: i) If  $\text{SI} < 0$  then the system is considered undersaturated with respect to the solid; ii) for  $\text{SI} = 0$  the system is in equilibrium with the solid and iii) for  $\text{SI} > 0$  the system is oversaturated with respect to the solid (MINEQL+, 2003). Growth experiment was modeled for 50 mM  $\text{Fe}^{2+}$  and 10 mM  $\text{PO}_4\text{-P}$  in presence of  $C_{\text{T,CO}_3} = 27$  mM. Septic experiment was modeled for 10 mM  $\text{Fe}^{2+}$  and 0.306 mM  $\text{PO}_4\text{-P}$  in presence of  $C_{\text{T,CO}_3} = 2.43$  mM which represents 100 mg/L  $\text{CaCO}_3$  alkalinity.

#### 4.3.8 Analytical methods:

The aqueous phase pH was measured by Semi Micro pH probe (Thermo Scientific Inc., Pittsburgh, PA). Conventional wastewater parameter chemical oxygen demand (COD) was analyzed by standard methods (Greenberg et al, 1992). Total iron (ferric and ferrous) in the solid phase and soluble  $\text{Fe}^{2+}$  were quantified using the Ferrozine assay (Stookey, 1970). Briefly, total iron was extracted in 0.5N HCl and the resulting extract was chelated with Ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine);  $\text{Fe}^{2+}$  forms a strong purple color which can be determined at 562.0 nm. The  $\text{Fe}^{3+}$  portion of the extract was reduced with 6.25N hydroxylamine, and the  $\text{Fe}^{3+}$  portion was determined by difference of initial ferrous minus final ferrous concentration. Aqueous  $\text{Fe}^{2+}$  was quantified using the Ferrozine assay minus the second step.  $\text{PO}_4^{3-}$  was determined spectrophotometrically at 880 nm by using sulfuric acid, ascorbic acid, ammonium molybdate and potassium antimonyl tartrate (Murphy and Riley, 1962).  $\text{NO}_3^-$ , and  $\text{SO}_4^{2-}$  were analyzed in the Ion Chromatography (IC) using 0.1 mL of filtered sample.

## 4.4 Results

**4.4.1 Growth experiments:** *Geobacter metallireducens* incubated for 4 hrs began to reduce  $\text{Fe}^{3+}$  very slowly. During the growth period, the color of the medium changed from amber to dark green and then faded to become colorless at the end. Significant precipitates formation started at 30 hrs which was reflected in sharp increase in phosphorus removal. Initially the phosphorus was removed slowly and  $\text{Fe}^{2+}$  produced at small amount. At earlier stage of the experiment, phosphorus was most likely used for cell growth together with initiation of vivianite formation. The x-ray peaks were attributed for vivianite when sample was analyzed at the end of the experiment. No significant pH change was observed which stayed within 6.5 to 7 at the start

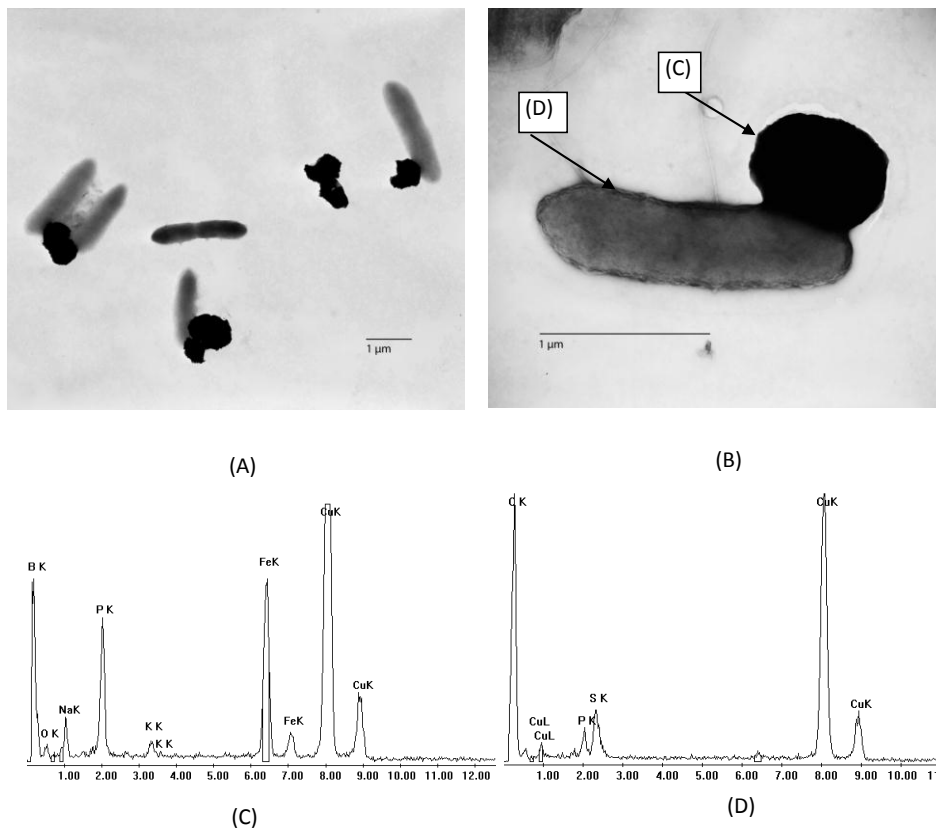


**Figure 14:**  $\text{Fe}^{2+}$  accumulation and removal of  $\text{PO}_4^{3-}$  during growth experiment with *Geobacter metallireducens* in 50 mM ferric citrate media. Results are the means of triplicate analyses; bars indicate one standard deviation. T-test (two-sample assuming unequal variances and 95% confidence interval) shows that  $\text{PO}_4^{3-}$  removal in presence of *Geobacter metallireducens* is significantly greater than no cells present at 96 hours for initial 14 mM  $\text{PO}_4^{3-}$ .

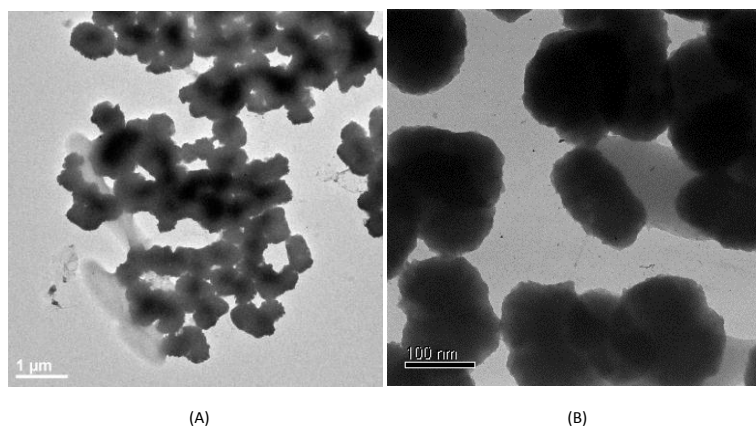
and end of experiment. During  $\text{Fe}^{3+}$  reduction; 50 mM  $\text{Fe}^{3+}$  bottles with phosphorus added initially was reduced to 25 mM. Whereas for  $\text{Fe}^{3+}$  bottles with no phosphorus addition;  $\text{Fe}^{3+}$  reduced approximately to 45 mM. Immediate addition of 10 mM  $\text{PO}_4\text{-P}$  at 30 hours decreased the phosphorus level to approximately 3 mM  $\text{PO}_4\text{-P}$  within 18 hrs from 30 to 48 hrs of the experiment (Fig. 14).

**4.4.2 Transmission electron microscopy (TEM):** TEM analysis was performed for centrifuges cell pellets collected from phosphate amended [FeCit (III) + P (added initially) + GS-15] and unamended sample [FeCit (III) + GS-15 with no additional phosphate addition] shown in Figure 15, 16, 17 and 18. TEM images reveal that precipitates grew or absorbed to the surface of the bacteria (Fig. 15). EDS response confirms that the precipitates have high content of iron and phosphorus. EDS response of bacteria itself was recorded with no presence of iron and phosphorus. The data suggests that the bacteria could work as nucleation site for mineral formation and precipitation. There were no precipitates found in the images of FeCit (III) + GS-15 samples with no additional phosphate addition. The TEM images at 48 hrs samples showed that the precipitated minerals grew in size due to adsorption in to each other. The presence of minerals were observed with the bacteria and in the solution (Fig. 16, 17, 18).

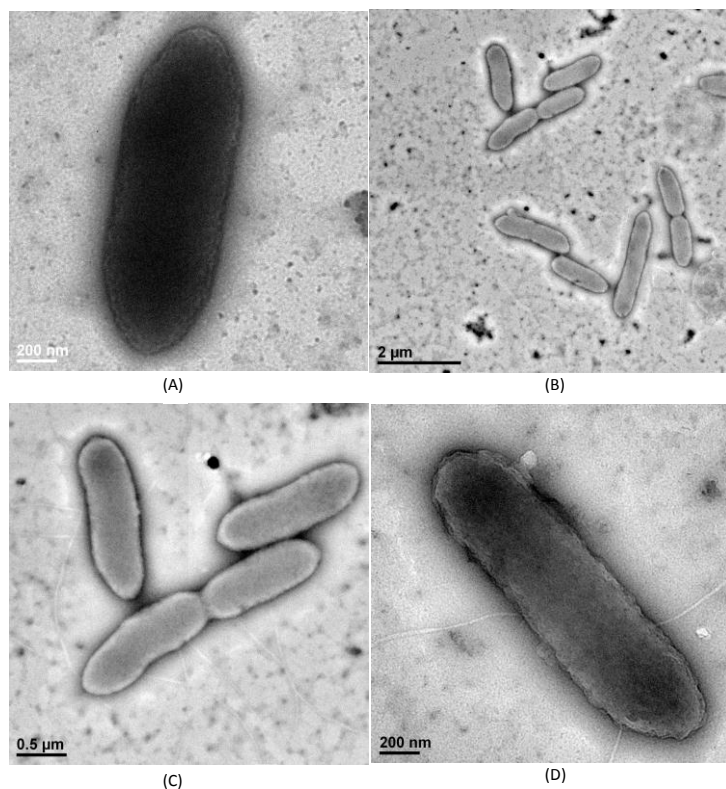
**4.4.3 X-Ray diffraction (XRD):** Both the filtered and dried precipitates of two types of sample i) phosphate added initially and ii) at 30 hrs showed that vivianite is the only mineral present in the sample (Fig. 19). The X-ray diffraction counts were overlaid with known iron minerals found in the literature. Only the known peaks of vivianite minerals match perfectly with the observed X-ray peaks of the samples. It confirms the presence and precipitation of only mineral vivianite in the sample. The observed response of un-amended phosphate sample did not show peaks with any known iron or any other type of minerals (Fig. 32).



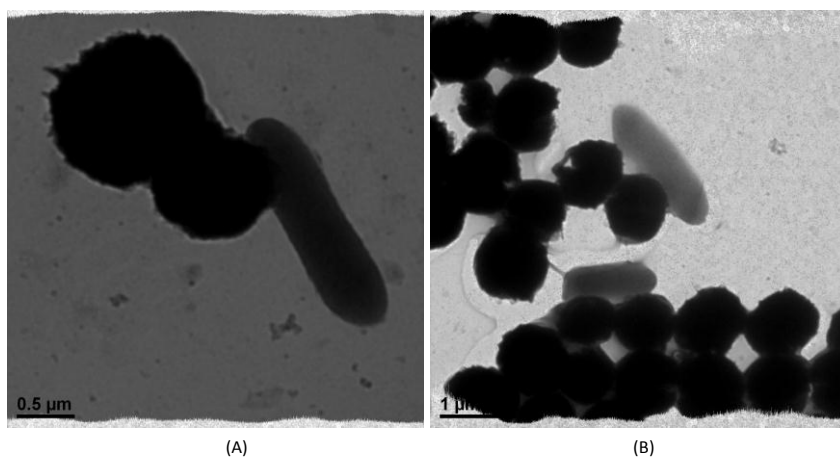
**Figure 15:** (A), (B) TEM images shows presence of bacteria and iron precipitates at 30 hrs in ferric citrate growth media with GS-15 and orthophosphate added initially in the experimental tubes. It shows precipitates formed on the surface of bacteria. (C) EDS response shows presence of Fe and P in the precipitates. (D) EDS response shows bacteria (cells) showing no presence of Fe and low P.



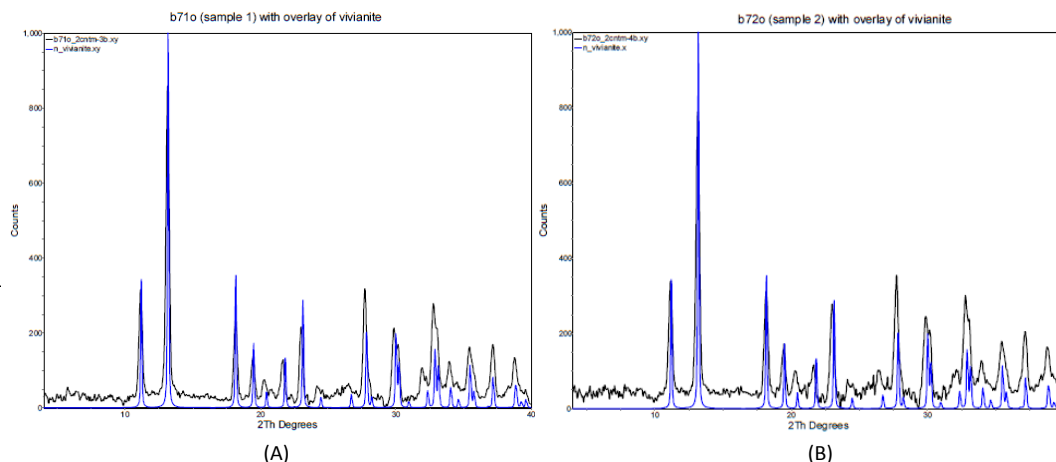
**Figure 16:** (A), (B) TEM images shows presence of bacteria and iron precipitates at 30 hrs in ferric citrate growth media with GS-15 and orthophosphate added initially in the experimental tubes. It shows precipitates formed on the surface of bacteria and in the solution.



**Figure 17:** (A), (B), (C), (D) TEM images shows presence of bacteria at 30 hrs in ferric citrate growth media with GS-15 and no orthophosphate added initially in the experimental tubes. It shows only the presence of bacteria and no precipitates were present.



**Figure 18:** (A), (B) TEM images shows presence of bacteria and iron precipitates at 48 hrs in ferric citrate growth media with GS-15 and orthophosphate added initially in the experimental tube. It shows precipitates formed on the surface of bacteria and in the solution.



**Figure 19:** Precipitates response from x-ray (black) overlaid with known vivianite response (blue) from (A) growth experiment with GS-15 and orthophosphate added initially in the experimental tube (B) growth experiment with GS-15 and orthophosphate added at 30 hrs in the experimental tube. Both responses from samples show exact matches with known vivianite response. No other mineral was found.

#### 4.4.4 Diluted wastewater experiment (batch 01):

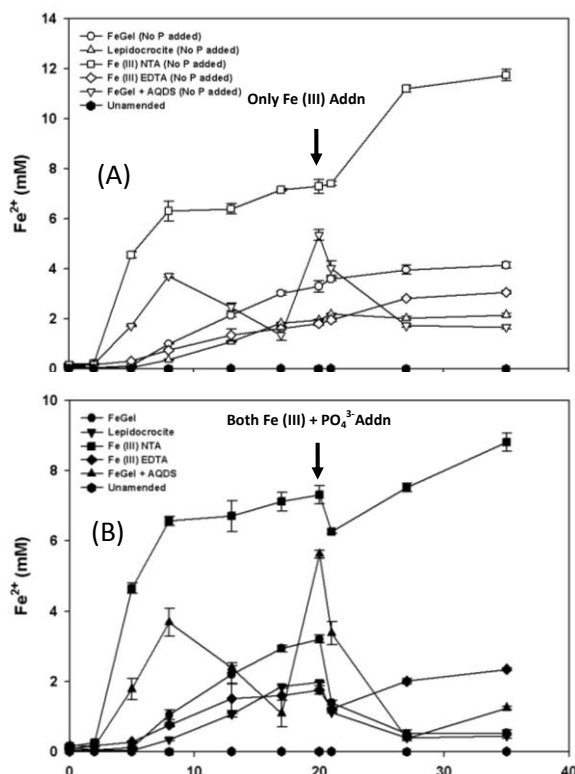
As the  $\text{PO}_4^{3-}$  was very low initially; additional 62 mg/L  $\text{PO}_4^{3-}$  was added to different iron amendment and un-amended sample with additional 12 mM of  $\text{Fe}^{3+}$  at day 20. To illustrate the effect of  $\text{PO}_4^{3-}$  in the aqueous  $\text{Fe}^{2+}$  present after 20 days of iron reduction in the solution; only  $\text{Fe}^{3+}$  added in the similar experimental bottles with no  $\text{PO}_4^{3-}$  addition. The effect of phosphate addition was distinctly identified as the soluble  $\text{Fe}^{2+}$  reduced sharply within the stoichiometric range of  $\text{PO}_4^{3-}$  requirement for vivianite formation within a day (Fig. 20). The phosphate data corroborated the findings as the added phosphate reduced significantly within one day period. The greatest iron reduction was observed for  $\text{Fe}^{3+}$  NTA, ferrihydrite and ferrihydrite + AQDS amended sample (Fig. 20). The pH varied within 5 and 7 at the start and end of experiment. The initial diluted wastewater COD was approximately 550 mg/L.  $\text{Fe}^{3+}$  NTA, Ferrihydrite and Ferrihydrite + AQDS amended samples showed significant COD removal potential ranging from 216.5, 333.8 and 286.3 mg/L (Table 8: Appendix)

The maximum  $\text{Fe}^{2+}$  accumulated in  $\text{Fe}^{3+}$  NTA amended sample i) approximately 7 mM for initial 20 mM iron amendment at 20 days. After phosphate addition and no phosphate addition at 20 day sample with 12 mM additional iron for both types,  $\text{Fe}^{2+}$  increased to 12 mM in the phosphate un-amended  $\text{Fe}^{3+}$  NTA sample. After initial dip of  $\text{Fe}^{2+}$  in the phosphate amended sample, the  $\text{Fe}^{2+}$  increased to 9 mM in the solution at 35 days of experimental period. The  $\text{Fe}^{2+}$  accumulation for other  $\text{Fe}^{3+}$  amended sample at the end of experimental period were the following: 3.75 mM (ferrihydrite with no P), 2.25 mM (ferrihydrite with P), 3 mM (lepidocrocite with no P), 0.75 mM (lepidocrocite with P), 3 mM (FeEDTA with no P), 0.8 mM (FeEDTA with P), 1.8 mM (Ferrihydrite + AQDS with no P) and 1.9 mM (Ferrihydrite + AQDS with P) (Fig. 20). The ferrihydrite + AQDS sample showed highest  $\text{Fe}^{2+}$  accumulation of 5 mM at day 20 before addition of phosphate in the system. The iron un-amended sample did not show any accumulation of  $\text{Fe}^{2+}$  as the onsite wastewater was deficient of  $\text{Fe}^{2+}$ .

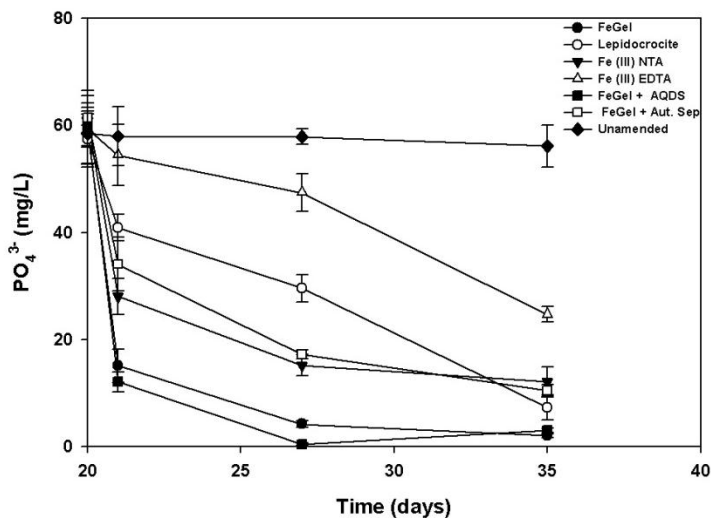
Phosphate removal was significant in ferrihydrite and ferrihydrite + AQDS amended sample. The phosphate concentration (initial concentration was 62 mg/L) reduced to 1.5 mg/L in those two incubation within 15 days of phosphate amendment (Fig. 21). Other phosphate data at experimental end points are 6 mg/L for lepidocrocite, 18 mg/L for  $\text{Fe}^{3+}$  NTA and 25 mg/L for  $\text{Fe}^{3+}$  EDTA. The phosphate concentration of unamended sample stayed around 60 mg/L. The phosphate removal of lepidocrocite amendment significantly contributed by  $\text{Fe}^{2+}$ -phosphate precipitation as the adsorption of phosphate to lepidocrocite was minimum confirmed by later experiment (data not shown). Though 50 to 70% phosphate removal was observed in  $\text{Fe}^{3+}$  EDTA and  $\text{Fe}^{3+}$  NTA sample, complete removal of phosphate was not observed possibly because of strong affinity of chelating agents for  $\text{Fe}^{2+}$ . EDTA is more recalcitrant compare to NTA and



possibly it contributed to higher phosphate in NTA amended sample compare to EDTA amended sample.



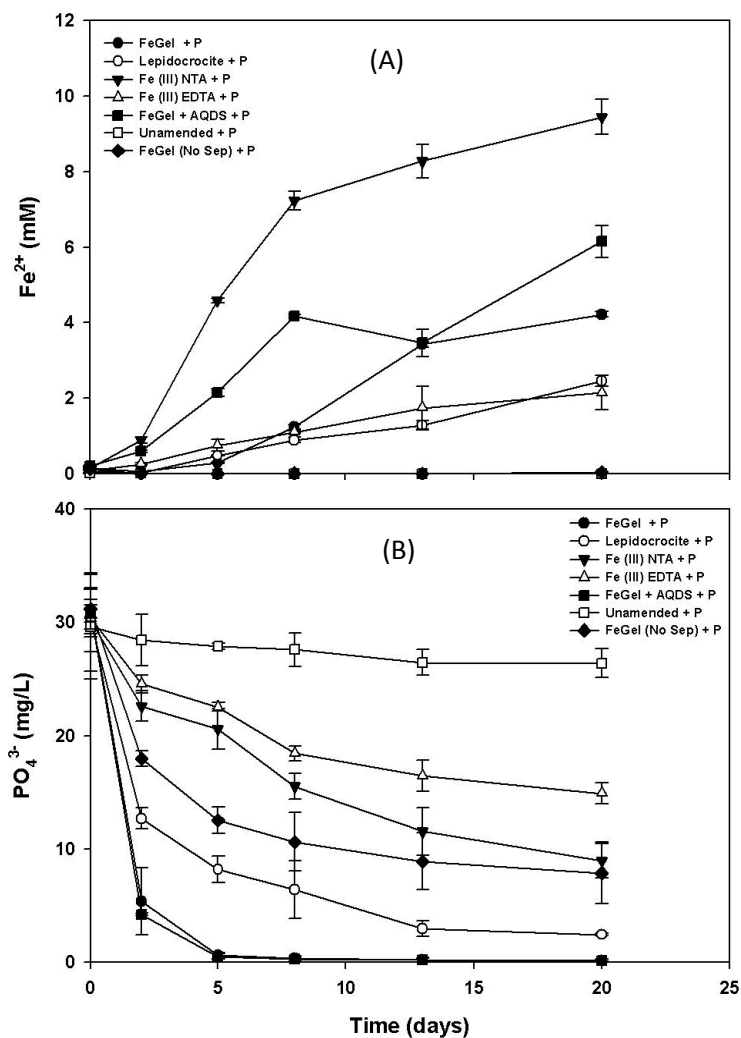
**Figure 20:** Accumulation of  $\text{Fe}^{2+}$  due to different types of  $\text{Fe}^{3+}$  amendment in diluted septic wastewater (batch 01). Experiment started with 20 mM  $\text{Fe}^{3+}$ . (A) Additional 10 mM  $\text{Fe}^{3+}$  (B) Additional 10 mM  $\text{Fe}^{3+}$  and 62 mg/L  $\text{PO}_4^{3-}$  were added at time = 20 days. Results are the means of triplicate analyses; bars indicate one standard deviation. Results are the means of triplicate analyses; bars indicate one standard deviation.



**Figure 21:** Removal of  $\text{PO}_4^{3-}$  for different types of  $\text{Fe}^{3+}$  amendment in 25 times diluted septic wastewater. Experiment started with 20 mM  $\text{Fe}^{3+}$ . Additional 10 mM  $\text{Fe}^{3+}$  and 62 mg/L  $\text{PO}_4^{3-}$  were added at 20 days. Results are the means of triplicate analyses; bars indicate one standard deviation. T-test (two-sample assuming unequal variances and 95% confidence interval) shows that  $\text{PO}_4^{3-}$  removal in presence of ferric iron is significantly greater than no ferric iron amendment samples at 35 days for 62 mg/L  $\text{PO}_4^{3-}$  were added at 20 days.

#### 4.4.5 Diluted wastewater experiment (batch 02):

Similar effects on phosphate removal and  $\text{Fe}^{2+}$  accumulation were observed when similar experiment as of batch 01 was run with the exception of 31 mg/L phosphate addition at the start of the experiment. The maximum  $\text{Fe}^{2+}$  accumulated in  $\text{Fe}^{3+}$  NTA amended sample i) approximately 9.8 mM for initial 20 mM iron amendment at 20 days. The  $\text{Fe}^{2+}$  accumulation for other  $\text{Fe}^{3+}$  amended sample at the end of experimental period were the following: 4.0 mM (ferrihydrite), 2.5 mM (lepidocrocite), 2.1 mM (FeEDTA), 6 mM (Ferrihydrite + AQDS). The iron unamended sample did not show any accumulation of  $\text{Fe}^{2+}$  as the onsite wastewater was deficient of  $\text{Fe}^{2+}$ . Phosphate removal was significant in ferrihydrite and ferrihydrite + AQDS amended sample as observed in batch 01 diluted experiment. The phosphate concentration (initial concentration was 31 mg/L) reduced to 0.2 mg/L in those two incubation within 20 days of phosphate amendment. Other phosphate data at experimental end points are 3 mg/L for lepidocrocite, 15 mg/L for  $\text{Fe}^{3+}$  NTA and 18 mg/L for  $\text{Fe}^{3+}$  EDTA. The phosphate concentration of unamended sample stayed around 30 mg/L (Fig. 22).

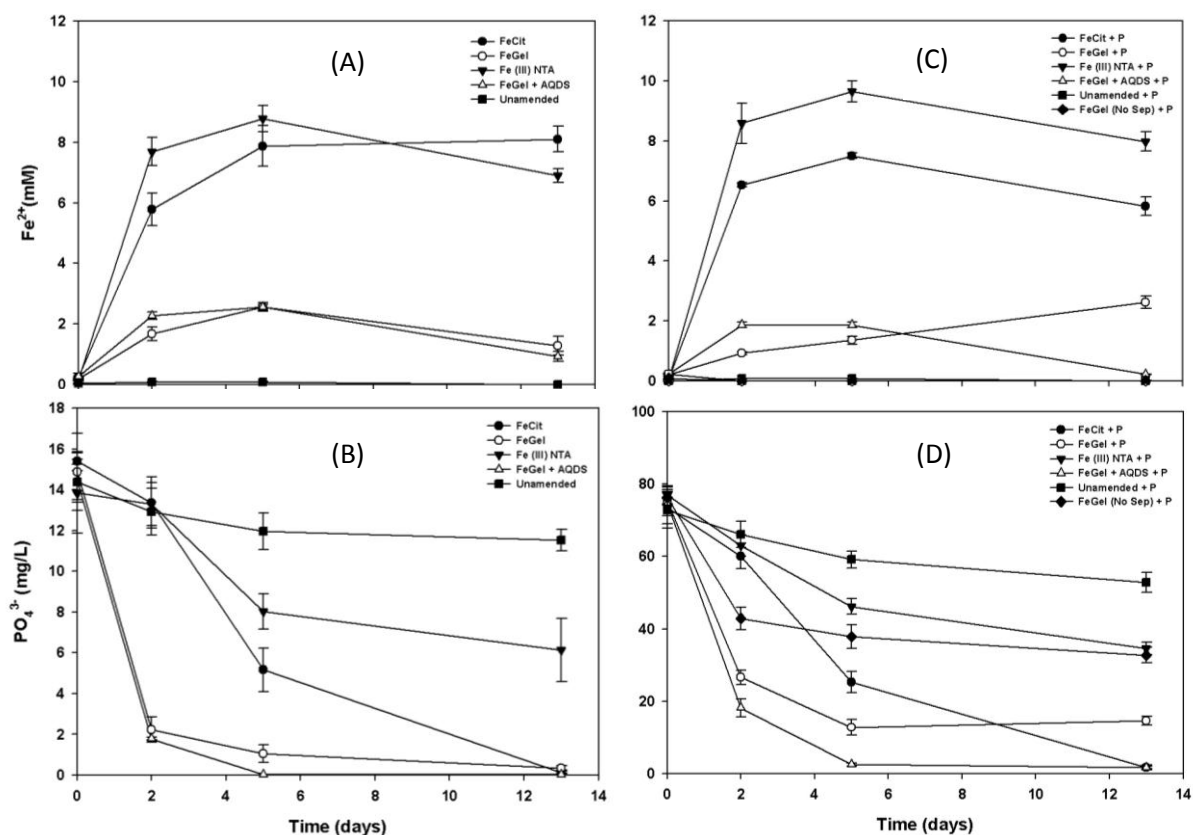


**Figure 22:** Accumulation of  $\text{Fe}^{2+}$  and removal of phosphate due to different types of  $\text{Fe}^{3+}$  amendment in 25 times diluted septic wastewater (batch 02). Experiment started with 20 mM  $\text{Fe}^{3+}$  and 31 mg/L  $\text{PO}_4^{3-}$ . Results are the means of triplicate analyses; bars indicate one standard deviation. T-test (two-sample assuming unequal variances and 95% confidence interval) shows that  $\text{PO}_4^{3-}$  removal in presence of ferric iron is significantly greater than no ferric iron amendment samples at 20 days for 31 mg/L  $\text{PO}_4^{3-}$  were added at day 0.

#### 4.4.6 Wastewater experiment (undiluted):

The maximum  $\text{Fe}^{2+}$  accumulated in  $\text{Fe}^{3+}$  NTA and  $\text{Fe}^{3+}$  citrate amended sample approximately to 7.8 and 8.1 mM after 13 days of amendment to the original wastewater. A separate set of experiment where 62 mg/L additional phosphate was added initially, the maximum  $\text{Fe}^{2+}$  accumulated in  $\text{Fe}^{3+}$  NTA and  $\text{Fe}^{3+}$  citrate amended sample were approximately to 8.1 and 6.1 mM at similar time frame. The  $\text{Fe}^{2+}$  accumulation in ferrihydrite amended sample was 2 mM and 3 mM for no phosphorus and phosphorus amended sample. Similar trend was observed for ferrihydrite + AQDS amended sample as the end point  $\text{Fe}^{2+}$  accumulation reported 1 mM and 0.5 mM. The iron unamended sample did not show any accumulation of  $\text{Fe}^{2+}$  as the onsite wastewater was deficient of  $\text{Fe}^{2+}$  (Fig. 23).

Phosphate removal was significant in ferric citrate, ferrihydrite and ferrihydrite + AQDS amended sample both for no P and with P amended samples. The phosphate concentration for no P addition sample (initial concentration was 15 mg/L) reduced to ~ 0 mg/L in those three incubation within 13 days of experimental period. Similar behavior was observed for phosphate added sample reducing phosphate concentration from approximately 80 mg/L to ~0 mg/L. 50% phosphate removal was observed for  $\text{Fe}^{3+}$  NTA amended sample for both conditions (no P addition and additional P addition). Approximately 25% phosphate removed from in the no ferric amended samples due to adsorption to the surface of the sludge (Fig. 23). The pH values did not change much and stayed within 6 to 7 range due to well buffered wastewater sample.



**Figure 23:** Accumulation of  $\text{Fe}^{2+}$  and removal of phosphate due to different types of  $\text{Fe}^{3+}$  amendment in septic wastewater. Experiment started with 10 mM  $\text{Fe}^{3+}$  with no external  $\text{PO}_4^{3-}$  addition (A, B) and with external 62 mg/L  $\text{PO}_4^{3-}$  (C, D). Results are the means of triplicate analyses; bars indicate one standard deviation. T-test (two-sample assuming unequal variances and 95% confidence interval) shows that  $\text{PO}_4^{3-}$  removal in presence of ferric iron is significantly greater than no ferric iron amendment samples at 13 days for in-situ  $\text{PO}_4^{3-}$  and 62 mg/L  $\text{PO}_4^{3-}$  added at day 0.

#### 4.4.7 Oxidation of $^{14}\text{C}$ -labeled carbon compounds in $\text{Fe}^{3+}$ -amended septic material:

The carbon molecules used in this experiment represent key carbon intermediates (the acetic acid), carbohydrate polymers (starch and xylose), and fats (oleic acid). Mineralization of four (4) different  $^{14}\text{C}$ -radiolabeled carbon molecules was monitored over the short term (48 hours), and long term (192 to 480 hours).

**4.4.7a Acetate.** Short term mineralization of acetate was most significantly enhanced by all ferric iron amendment except for ferrihydrite with AQDS amended sample (Figure 24a). Long term

acetate mineralization was increased by ferrihydrite, lepidocrocite, ferric NTA and ferric EDTA, relative to un-amended samples (Figure 24b). The maximum short term mineralization was 33.9% and 27.48% for  $\text{Fe}^{3+}$  NTA and ferrihydrite amended compare to 13.2% for un-amended sample (Table 5). The maximum mineralization of acetate was approximately 84.3% in ferric NTA, 76.8% in ferrihydrite and 79.9% in ferrihydrite + AQDS sample. There is no significant production of methane in ferric amended or un-amended sample. Probably methanogenesis was not the dominant pathway present in the wastewater.

**4.4.7b Xylose.** Short term mineralization of xylose was most significantly enhanced by  $\text{Fe}^{3+}$  NTA, ferrihydrite and  $\text{Fe}^{3+}$  EDTA amended sample (Figure 25a). Long term starch mineralization was increased by ferrihydrite, lepidocrocite, ferric NTA, ferric EDTA and ferrihydrite + AQDS relative to un-amended samples (Table 06). The maximum short term mineralization was 20.1% .19.6% and 15% for  $\text{Fe}^{3+}$  NTA, ferrihydrite and FeEDTA amended compare to 11.1% for un-amended sample. The maximum mineralization of xylose was approximately 41.8% in ferric NTA, 48.4% in ferrihydrite and 56.8% in ferrihydrite + AQDS sample. There is no significant production of methane in ferric amended or un-amended sample.

**4.4.7c Starch.** Short term mineralization of starch was most significantly enhanced by  $\text{Fe}^{3+}$  NTA, ferrihydrite and ferrihydrite + AQDS amended samples (Figure 26a). Long term starch mineralization was increased by ferrihydrite, lepidocrocite, ferric NTA, ferric EDTA and ferrihydrite + AQDS relative to un-amended samples (Table 6). The maximum short term mineralization was 37.1%, 21.2% and 22.8% for  $\text{Fe}^{3+}$  NTA, ferrihydrite and ferrihydrite + AQDS amended compare to 15.3% for un-amended sample. The maximum mineralization of starch was significantly higher (65.9% to 94.6%) in ferric amended sample compare to un-

amended sample (51.4%). There is no significant production of methane in ferric amended or un-amended sample.

**4.4.7d Lipid.** Short term mineralization of lipid was most significantly enhanced by Fe<sup>3+</sup> NTA, and lepidocrocite amended samples (Figure 27a). Long term lipid mineralization was significantly increased by ferrihydrite, lepidocrocite, ferric NTA, ferrihydrite + AQDS relative to un-amended samples (Table 6). The maximum short term mineralization was 38.5%, 33.1%, 24.2% and 20.3% for Fe<sup>3+</sup> NTA, lepidocrocite, ferrihydrite and FeEDTA amended compare to 15.3% for un-amended sample. The maximum mineralization of lipid was significantly higher (ranging from 62.6% to 90.1%) in ferric amended sample (except EDTA) compare to un-amended sample (51.4%). There is no significant production of methane in ferric amended or un-amended sample.

**Table 5:** Maximum % of <sup>14</sup>C carbon molecules recovered as CO<sub>2</sub> within 48 hrs

Iron Forms	Maximum % of <sup>14</sup> C Carbon Oxidized to <sup>14</sup> CO <sub>2</sub>							
	Acetate		Xylose		Starch		Lipid	
	CO <sub>2</sub> (SD <sup>1</sup> )	H <sup>2</sup>	CO <sub>2</sub> (SD <sup>1</sup> )	H <sup>2</sup>	CO <sub>2</sub> (SD <sup>1</sup> )	H <sup>2</sup>	CO <sub>2</sub> (SD <sup>1</sup> )	H <sup>2</sup>
<b>Ferrihydrite</b>	27.48 (4.58)	Yes	19.61 (4.99)	No	21.16 (1.88)	Yes	24.16 (2.49)	Yes
<b>Lepidocrocite</b>	20.35 (3.68)	Yes	6.02 (6.06)	No	15.25 (2.24)	No	33.07 (1.96)	Yes
<b>Fe<sup>3+</sup> NTA</b>	33.85 (7.61)	Yes	20.13 (6.47)	No	37.05 (2.85)	Yes	38.50 (2.34)	Yes
<b>Fe<sup>3+</sup> EDTA</b>	20.48 (3.76)	Yes	15.02 (7.81)	No	19.11 (1.17)	Yes	20.25 (2.65)	No
<b>Ferrihydrite + AQDS</b>	12.44 (1.74)	No	5.37 (2.01)	No	22.79 (0.53)	Yes	15.31 (0.68)	No
<b>Un-Amended System</b>	13.23 (2.85)	N/A	11.13 (5.06)	N/A	15.30 (1.09)	N/A	15.75 (3.88)	N/A

\*1: SD = Standard Deviation

\*2: H = Hypothesis that oxidation to CO<sub>2</sub> due to iron amendment is significantly greater than no iron amendment oxidation (95% confidence interval: using t-Test: Two-sample assuming unequal variances)

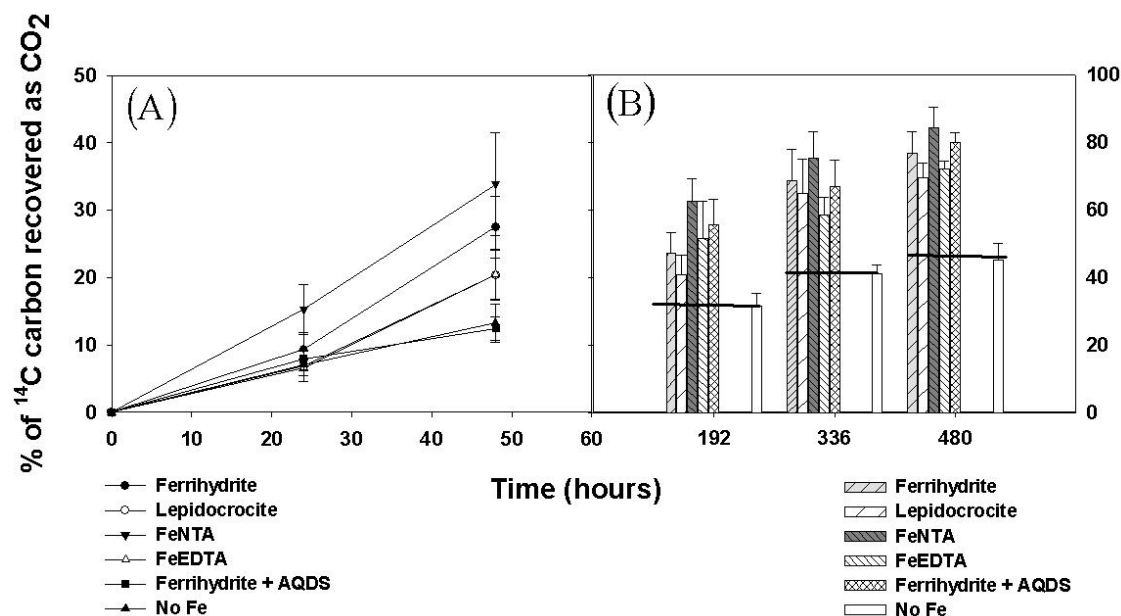


**Table 6:** Maximum % of  $^{14}\text{C}$  carbon molecules recovered as  $\text{CO}_2$  within 480 hrs

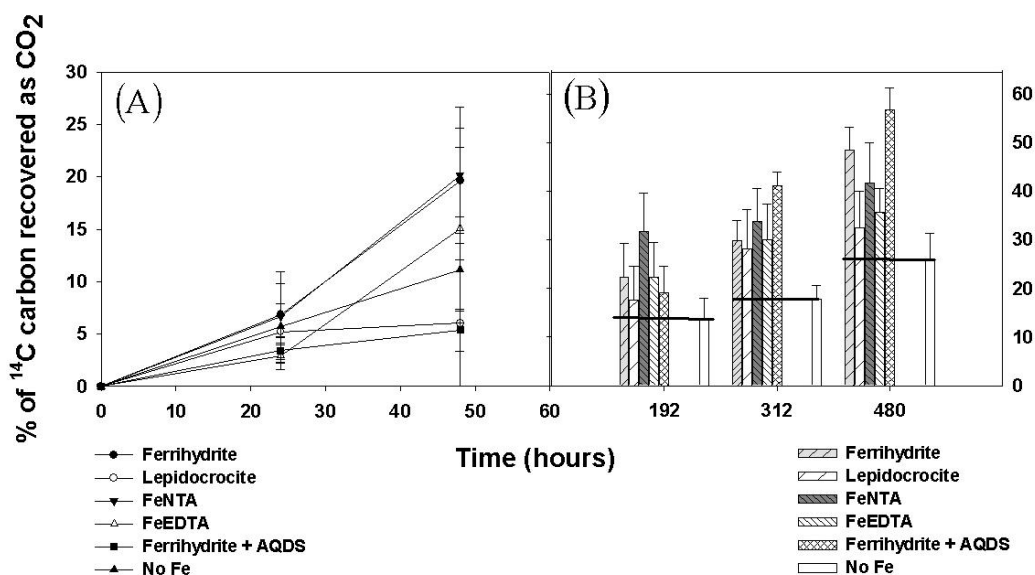
Iron Forms	Maximum % of $^{14}\text{C}$ Carbon Oxidized to $^{14}\text{CO}_2$							
	Acetate		Xylose		Starch		Lipid	
	$\text{CO}_2$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CO}_2$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CO}_2$ (SD <sup>1</sup> )	H <sup>2</sup>	$\text{CO}_2$ (SD <sup>1</sup> )	H <sup>2</sup>
<b>Ferrihydrite</b>	76.75 (6.52)	Yes	48.41 (4.89)	Yes	84.18 (6.27)	Yes	66.51 (3.56)	Yes
<b>Lepidocrocite</b>	69.48 (4.42)	Yes	32.56 (7.44)	Yes	90.60 (5.45)	Yes	62.55 (3.62)	Yes
<b>Fe<sup>3+</sup> NTA</b>	84.26 (6.98)	Yes	41.78 (8.24)	Yes	94.62 (8.27)	Yes	90.05 (3.32)	Yes
<b>Fe<sup>3+</sup> EDTA</b>	72.11 (2.35)	Yes	35.79 (4.84)	Yes	65.88 (4.37)	Yes	46.57 (4.13)	No
<b>Ferrihydrite + AQDS</b>	79.86 (2.85)	Yes	56.81 (4.53)	Yes	77.62 (4.26)	Yes	78.07 (4.43)	Yes
<b>Un-Amended System</b>	45.31 (4.94)	N/A	25.86 (5.46)	N/A	51.42 (5.05)	N/A	44.62 (5.95)	N/A

\*1: SD = Standard Deviation

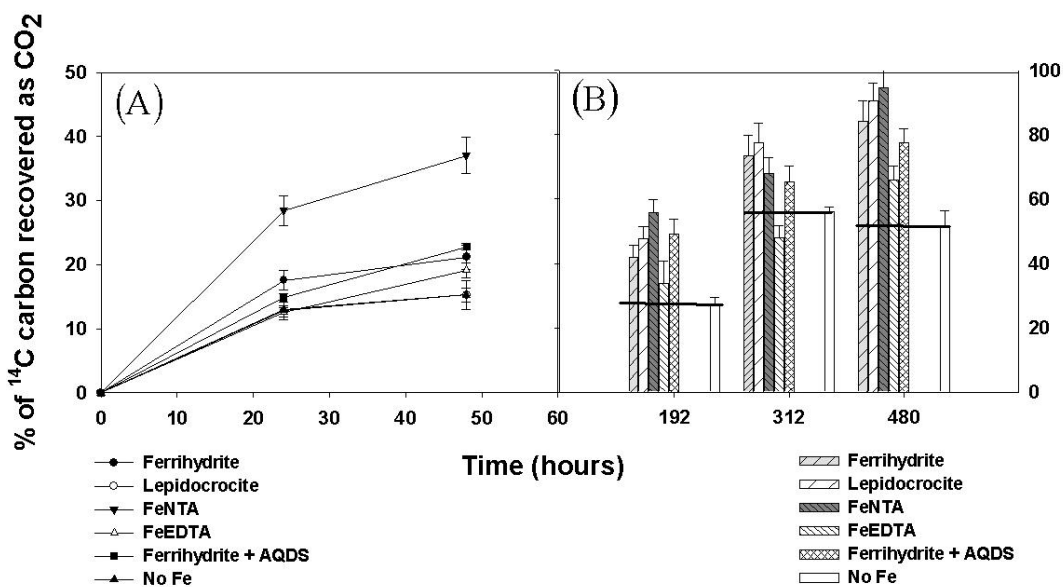
\*2: H = Hypothesis that oxidation to  $\text{CO}_2$  due to iron amendment is significantly greater than no iron amendment oxidation (95% confidence interval: using t-Test: Two-sample assuming unequal variances)



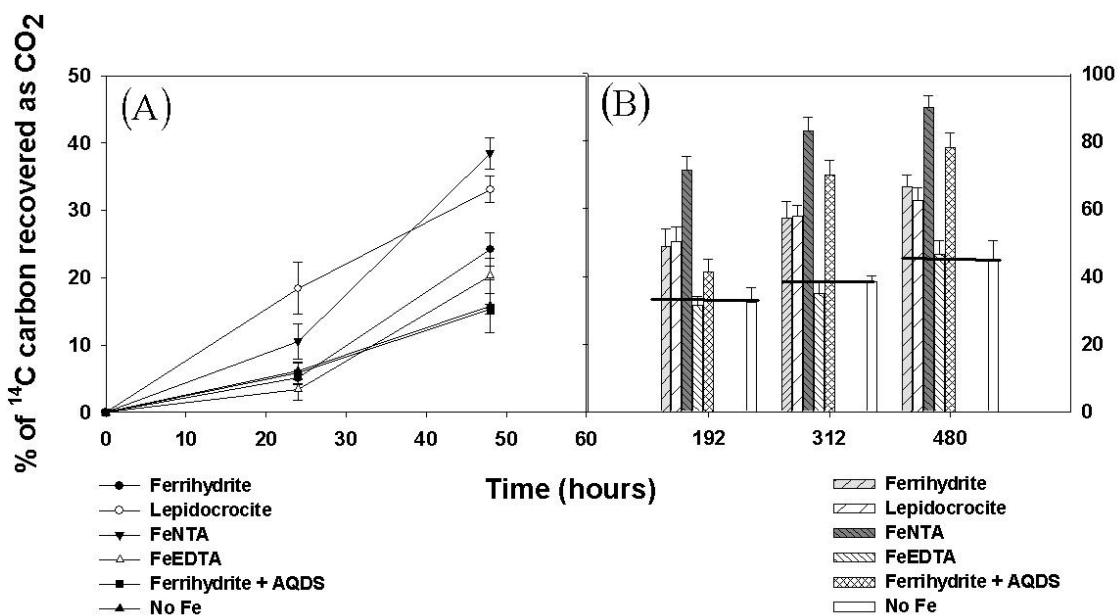
**Figure 24:**  $^{14}\text{C}$  acetate recovered as  $\text{CO}_2$  in  $\text{Fe}^{3+}$  amended and un-amended septic wastewater. Results are the means of triplicate analyses; bars indicate one standard deviation. 10 mL triplicate anaerobic samples of septic wastewater + 10 mM iron forms (five types) + 0.1 mL  $^{14}\text{C}$  acetate were incubated in 28 mL anaerobic pressure tubes.



**Figure 25:**  $^{14}\text{C}$  xylose recovered as  $\text{CO}_2$  in  $\text{Fe}^{3+}$  amended and un-amended septic wastewater. Results are the means of triplicate analyses; bars indicate one standard deviation. 10 mL triplicate anaerobic samples of septic wastewater + 10 mM iron forms (six types) + 0.1 mL  $^{14}\text{C}$  xylose were incubated in 28 mL anaerobic pressure tubes.



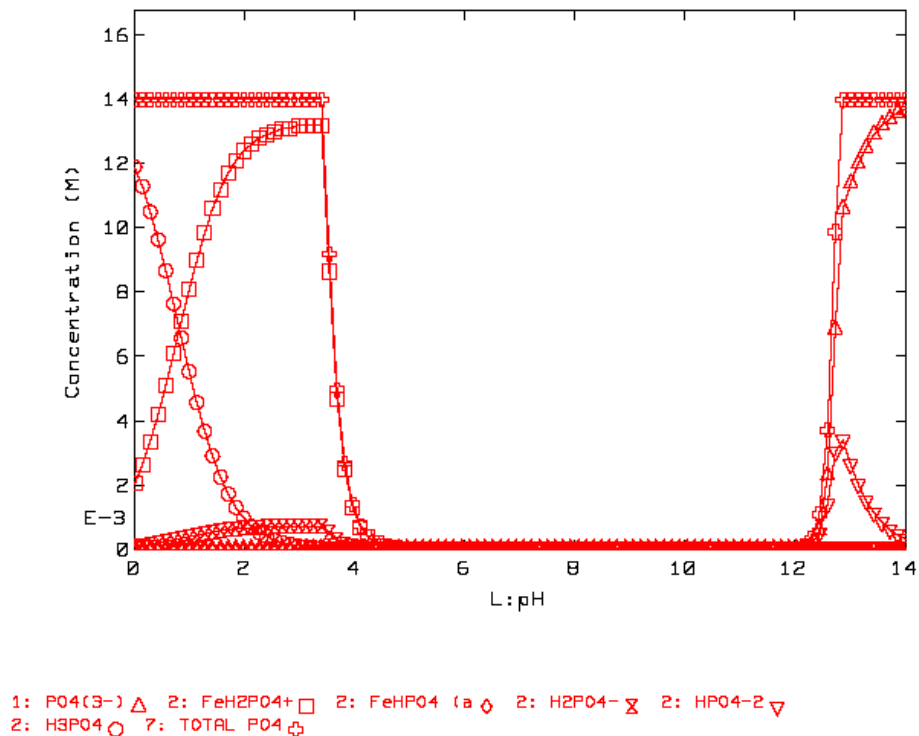
**Figure 26:**  $^{14}\text{C}$  starch recovered as  $\text{CO}_2$  in  $\text{Fe}^{3+}$  amended and un-amended septic wastewater. Results are the means of triplicate analyses; bars indicate one standard deviation. 10 mL triplicate anaerobic samples of septic wastewater + 10 mM iron forms (six types) + 0.1 mL  $^{14}\text{C}$  starch were incubated in 28 mL anaerobic pressure tubes.



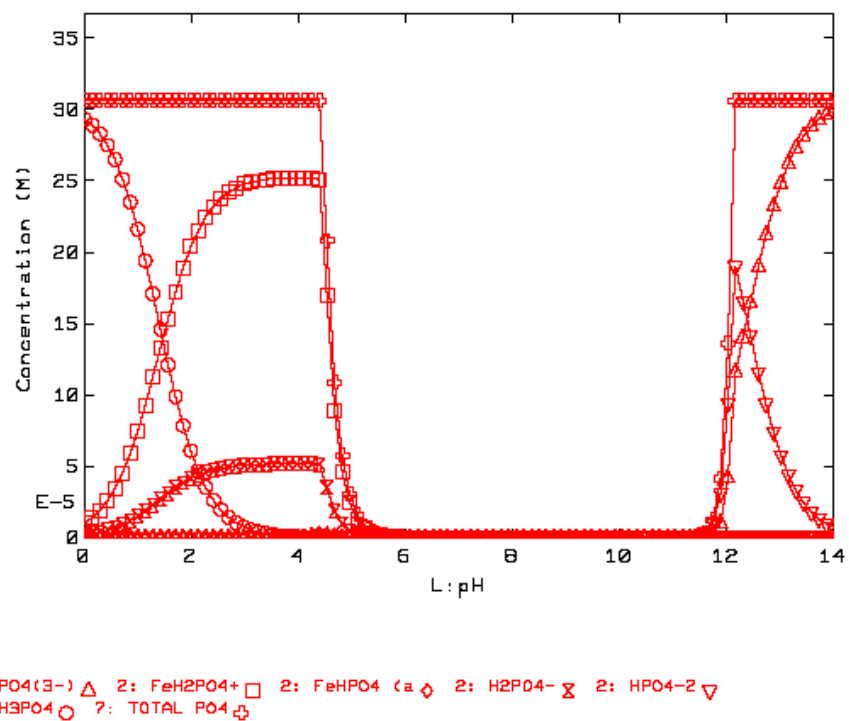
**Figure 27:**  $^{14}\text{C}$  lipid recovered as  $\text{CO}_2$  in  $\text{Fe}^{3+}$  amended and un-amended septic wastewater. Results are the means of triplicate analyses; bars indicate one standard deviation. 10 mL triplicate anaerobic samples of septic wastewater + 10 mM iron forms (six types) + 0.1 mL  $^{14}\text{C}$  lipid were incubated in 28 mL anaerobic pressure tubes.

#### 4.4.8 MINEQL+ : growth and wastewater experiment modeling:

Equilibrium modeling of growth experiment in ferric citrate media confirmed that vivianite is the dominant mineral that could be precipitated in growth experiment. Saturation indices indicated that the solution is oversaturated ( $SI > 0$ ) with respect to vivianite from pH 3.5 to pH 12 (**Fig. 33**). Equilibrium modeling of septic experiment in septic experiment confirmed that vivianite is the dominant mineral that could be precipitated in the sludge. Saturation indices indicated that the solution is oversaturated ( $SI > 0$ ) with respect to vivianite from pH 4.25 to pH 11.5 (**Fig. 36**). Figures 28 and 29 shows  $PO_4^{3-}$  speciation in ferric citrate and septic wastewater samples.



**Figure 28:**  $PO_4$  speciation diagram: MINEQL+ modeling (50 mM  $Fe^{2+}$  and 10 mM  $PO_4$ -P in presence of  $C_{T,CO_3} = 27$  mM-considering vivianite present).



**Figure 29:**  $\text{PO}_4^{3-}$  speciation diagram: MINEQL+ modeling (10 mM  $\text{Fe}^{3+}$  and 0.306 mM  $\text{PO}_4^{3-}\text{-P}$  in presence of  $C_{\text{T}, \text{CO}_3} = 2.43$  mM considering vivianite present in septic wastewater).

## 4.5 Discussion

Growth experiment showed removal of phosphorus as vivianite in presence of iron reducing microorganisms *Geobacter metallireducens*. It corresponded to previous studies on growth experiment using *Shewanella Putrefaciens* (Jorand et al, 2000). The precipitates' XRD intensity indicated the presence of similar shape and size of crystals (vivianite) precipitated biotically (phosphate added initially before iron reduction started) and abiotically (phosphate added after significant accumulation of  $\text{Fe}^{2+}$ ) during the growth experiment. Phosphorus was removed as vivianite (XRD and TEM data confirmed it) at a rate of 0.215 mM/hr when no  $\text{Fe}^{2+}$  was present initially from 0 to 48 hrs of the experiment. When significant  $\text{Fe}^{2+}$  was present due to iron reduction, the phosphorus was removed at a rate of 0.6 mM/hr from 30 hrs to 48 hrs in the growth experiment with GS-15. Previous studies showed phosphorus removal at a rate of 0.0645 mM/hr in growth experiment with *Shewanella Putrefaciens* in ferric citrate media (Jorard et al., 2000). Zachara et al., 1998 showed significant phosphorus removal from goethite+AQDS media in presence of *Shewanella Putrefaciens* at a rate of 0.0395 mM/hr. The difference in phosphate removal rate by *Geobacter metallireducens* and *Shewanella Putrefaciens* probably was dominated by the faster growth rate of *Geobacter metallireducens* in anaerobic media compare to *Shewanella Putrefaciens*. Previous studies on vivianite solubility product determination reported vivianite formation within 5 hrs (Nriagu, 1972) and 1 month (Singer, 1972) of the experimental period in strictly abiotic anaerobic conditions. No kinetic study dedicated to abiotic phosphate removal as vivianite is identified in the literature. But the vivianite formation kinetics in natural environment will be highly influenced by the in situ conditions of the water and wastewater such as pH, buffer, NOM, COD, ionic strength, alternate ions present, temperature, mixing conditions etc and thus might require longer time to form in original water

and wastewater conditions. Future studies on kinetic study of vivianite precipitation abiotically under varying environmental conditions can facilitate the basic understanding of the microbial vivianite precipitation and the role of microorganisms in the precipitation process.

TEM images showed that microorganisms might have been used as nucleation sites for crystal growth based on the amount of minerals aggregated around the microorganisms. The exact time for vivianite precipitation could not be determined either as the solid sample was collected at the end of experimental period. TEM-EDS images show that the precipitates (Fig 15c) showed abundant presence of iron and phosphorus which indicates that the mineral might be vivianite. In TEM, the area of interest is irradiated with a finely focused electron beam (typically <5 nm and at best < 0.1 nm in diameter). The types of signals produced from the interaction of electrons with the sample include secondary electrons, backscattered electrons, characteristic x-rays and other photons of various energies. The characteristic emitted x-rays can yield qualitative and quantitative elemental information from regions of a specimen nominally 1  $\mu\text{m}$  depth under normal operating conditions (Williams et al., 2009; Goldstein et al., 2007). Since, the bacteria size ranges from 1  $\mu\text{m}$  to 2  $\mu\text{m}$  in length and 0.5  $\mu\text{m}$  in diameter, EDS can identify the elemental composition of significant portion of the cell mass.

X-ray diffraction data confirmed that the only mineral present in experimental sample was vivianite. The EDS data of microorganism did not show any presence of iron and phosphorus which indicates that iron/phosphorus mineral or precipitates was not accumulated or produced inside the microorganisms (Fig 15d). Phosphate un-amended growth media did not show any presence of precipitates which was supported by minimum phosphate removal in phosphate un-amended sample in the growth experiment (Fig 14 and 17).

Laboratory studies have proven that cells can act as templates for the nucleation of metal precipitates (Langley and Beveridge, 1999), and there is evidence that minerals in natural environments nucleate on cells (Ferris et al., 1986, 1987; Fortin et al., 1998). In addition to expelling metal ions from the cell to form spatially distinct minerals, bacteria can sorb large amounts of metals and form surface precipitates. Bacteria usually have a net negative surface charge at circumneutral pH as a result of acidic functional groups (Beveridge, 1981; Collins and Stotzky, 1992). This makes them highly reactive to positively charged metal species, as demonstrated by studies of metal sorption (Fein et al., 1997; Daughney and Fein, 1998). The reactivity of the cell to metal ions at the bulk aqueous interface has several consequences. Sorption of metals to the outer membrane of gram-negative bacteria and subsequent mineral growth at the surface may compete with mineral formation in the bulk solution, or even disable the cells.

Septic experiment (diluted and undiluted) using ferric citrate, ferrihydrite, ferrihydrite + AQDS showed significant removal of phosphorus. The higher and quicker  $\text{Fe}^{2+}$  accumulation was observed in original wastewater sample due to the presence of abundant microorganism compare to diluted sample. Adsorption of phosphate onto metal oxide surfaces is the first step in the formation of iron phosphates (Swenson et al., 1949; Haseman et al., 1950). These minerals react with phosphate to form discrete iron phosphate phases if the reaction time is very long or if temperatures or phosphate levels are elevated (Swenson et al., 1949; Haseman et al., 1950; Bache, 1963). In the diluted wastewater experiment, the pH values went down at the end of the experiment possibly due to vivianite precipitation and generation of  $\text{H}^+$ .

Phosphate removal was significant in ferrihydrite and ferrihydrite + AQDS amended sample. The phosphate concentration (initial concentration was 62 mg/L) reduced to 1.5 mg/L in



those two incubation within 15 days of phosphate amendment (Fig. 21). Three important factors might have contributed to the significant phosphate removal i) precipitation of phosphate with aqueous  $\text{Fe}^{2+}$  possibly as vivianite (supported by phosphate data of septic + ferrihydrite and sterile septic + ferrihydrite) and ii) adsorption of phosphate in ferrihydrite matrix (supported phosphate data of sterile septic + ferrihydrite) iii) combination of these two-adsorption of phosphate and  $\text{Fe}^{2+}$  on the ferrihydrite surface and conversion of  $\text{Fe}^{2+}$  and phosphate to vivianite (hypothesized based on literature findings and observation of other ferric iron amendment in this study) (Fig. 21, 22 and 23).

Fe(hydr)oxide minerals added to cultures of *S. putrefaciens* have a demonstrated high affinity for the cell surface (Glasauer et al., 2001). The location of biomineral precipitates will depend on properties of the metal and the microorganisms, reflecting chemical gradients established between bacteria and the extracellular environment. Amorphous  $\text{Fe}^{2+}$   $\text{Fe}^{3+}$  hydroxide (known as “green rust”: metal oxides) is used by dissimilatory metal-reducing bacteria (DMRB) in anoxic environment to form vivianite (Zachara et al., 1998; Hansen and Poulsen, 1999). Presence of AQDS is also investigated in amorphous iron oxide reduction by DMRB *Shewanella putrefaciens*. Vivianite precipitation was reported within 11 days of incubation period (Zachara et al., 1998).

Enrichment culture in returned liquor of municipal wastewater treatment plant with iron reducing bacteria and a suspension of ferric hydroxide showed significant removal of phosphate (70 mg/L to 1 mg/L) with 10 days of incubation period (Ivanov et al., 2004). Column test with fixed-bed filters containing ferric oxyhydroxide media removed phosphate during 30 month of dosing with domestic wastewater. Reductive iron dissolution reactions delivered consistent moderate concentrations of Fe into solution (2.91.6 mg/L), and influent  $\text{PO}_4\text{-P}$  of (3.7+ 1.0

mg/L) was attenuated to  $0.09 \pm 0.04$  mg/L in the column effluent (98% removal) (Robertson and Lombardo, 2011). In our experiment; significant removal of phosphate was observed in ferric citrate, ferrihydrite and ferrihydrite + AQDS within 13 days of incubation removing phosphate approximately from 15.4 to 0.13 mg/L (ferric citrate), 14.9 mg/L to 0.35 mg/L (ferrihydrite) and 14.4 mg/L to 0.03 mg/L (ferrihydrite + AQDS). For high phosphate loading condition: phosphate removed approximately from 73.5 to 1.7 mg/L (ferric citrate), 75.2 mg/L to 14.7 mg/L (ferrihydrite) and 74.5 mg/L to 1.7 mg/L (Fig. 23).

Though phosphate removal in ferric NTA and ferric-EDTA sample were prominent; it was much lower than other iron amended sample. EDTA and NTA are very resistant to anaerobic degradation (Yuan and VanBriesen, 2008) and therefore will remain as ligands for  $\text{Fe}^{3+}$  in strictly anoxic systems (Lovley, 2006). Rates of bacterial  $\text{Fe}^{3+}$  reduction in the presence of dissolved chelating agents correlate with the thermodynamic stability constants of the  $\text{Fe}^{3+}$ -organic complexes, implying that chemical speciation governs  $\text{Fe}^{3+}$  bioavailability (Haas and Dichristina, 2002). Chelating ligands like EDTA and NTA have the potential to bind the iron so tightly that  $\text{Fe}^{3+}$  is not readily bioavailable to all  $\text{Fe}^{3+}$  reducers (Zhu et al, 2009). It might be possible that the  $\text{Fe}^{2+}$  was chelated with NTA and EDTA tightly and thus prevented phosphate removal from the system. Only degradation of NTA and EDTA can free the  $\text{Fe}^{2+}$  in the solution to bind with soluble phosphate and get removed from the system. Previously studied reduction kinetics of  $\text{Fe}^{3+}$  citrate,  $\text{Fe}^{3+}$  NTA and  $\text{Co}^{3+}$ EDTA in the presence of dissimilatory metal reducing bacteria (DMRB) showed that the reduction rates varies among chelating agent types with  $\text{Fe}^{3+}$  citrate >  $\text{Fe}^{3+}$  NTA >  $\text{Co}^{3+}$ EDTA (higher to lower) (Liu et al, 2002). MINEQL+ modeling simulating the growth and septic experiment confirmed that vivianite should be the dominant minerals present in equilibrium (Fig 28 and 29).

$\text{Fe}^{3+}$  amendments also increased the rate and extent of mineralization to  $\text{CO}_2$  over both the short and long term. The data showed that methanogenesis was not significant in unamended sample thus effect of iron reduction could not be validated for this onsite wastewater. Previous studies in this research showed that when methanogenesis is active, iron reduction significantly suppress methane production. Short term and long term carbon mineralization are both critical in septic systems. Carbon enters these systems primarily in the aqueous phase or as suspended solids (Crites and Tchobanoglous, 1998, Baumann et al, 1977) and the aqueous phase moves through the system in a matter of days, whereas the “sludge layer” remains for weeks and even months or possibly years (Crites and Tchobanoglous, 1998, Baumann et al, 1977). The short term mineralization data presented above demonstrate that ferric iron will increase the extent of mineralization within 30 hours of amendment, which will influence carbon that exists primarily in the aqueous phase as it moves through a septic tank. The long term mineralization data indicate that carbon that remains in the septic tank will be: a) mineralized to a greater extent as  $\text{CO}_2$  when  $\text{Fe}^{3+}$  is present.

Typical BOD, TSS and phosphorus concentration in raw domestic wastewater are reported as 155-286 mg/L (average 250 mg/L), 155-330 mg/L (average 250 mg/L) and 6-12 mg-P/L (average 10 mg/L) respectively (Lowe et al., 2007) in the supernatant. After undergoing through physical and biological treatment in a septic tank, the typical effluent characteristics have been reported as 118-189 mg/L BOD (average 120 mg/L), 36-85 mg/L TSS (average 60 mg/L) and 8.1-8.2 mg-P/L phosphorus (average 8.1 mg/L) in the effluent (Lowe et al, 2007; Crites and Tchobanoglous, 1998).  $\text{Fe}^{3+}$  NTA, Ferrihydrite and Ferrihydrite + AQDS amended samples showed significant COD removal potential ranging from 216.5, 333.8 and 286.3 mg/L (Table 8: Appendix) in septic experiment. Moreover,  $^{14}\text{C}$  oxidation data shows that complex

carbon molecules can be oxidized to  $\text{CO}_2$  in presence of  $\text{Fe}^{3+}$ . Significant phosphate (>95%) removal was observed for several ferric iron amendment. Thus oxidation and phosphate removal data clearly shows that iron reduction could be an effective strategy to meet stringent treatment goals by oxidizing complex forms of carbon and removing phosphate from wastewater.

## CHAPTER 5 CONCLUSION AND FUTURE WORK

The organic concentration of the influent waste stream has serious effects on an OWS. 30-50% of the  $BOD_5$  can be removed within the tank for conventional OWS utilizing a septic tank followed by soil treatment. Thus, high concentrations of organic material can be applied in soil treatment unit leading to cell growth. This cell growth can eventually reduce the soil infiltration capacity and can cause failure of the OWS (Lowe et al., 2007). Additionally, the settled sludge is decomposed biologically but often at an extremely slow rate that leads to sludge accumulation and a requirement for frequent cleaning (Crites and Tchobanoglous, 1998; Perkins, 1989; Parten, 2010). Thus, higher removal of  $BOD_5$  inside the septic tank is desirable for effective performance. In addition, many systems generate more  $CH_4$  than  $CO_2$ , which is a concern because methane is a more potent greenhouse gas than carbon dioxide (US EPA, 2009). Moreover, the need to control phosphorus loading from onsite wastewater to receiving waters and to prevent eutrophication has been well documented (Vollenweider, 1985, Crites and Tchobanoglous, 1998).

Our research demonstrate that adding ferric iron in various forms to septic wastewater increase the rate and extent of mineralization to  $CO_2$ , and limits the production of methane from a variety of carbon sources. Moreover, the reduced iron can remove phosphate effectively from the wastewater. This is critical because ferric iron can be easily added with minimal disruption to on site systems either directly (at the tank) or indirectly (added to drains/toilets on the system) if the form is soluble, of which several were. Proper iron types conducive to vivianite precipitation had been optimized in this research. This study also confirms that iron reduction increases microbial diversity and iron reducer biomass.

We believe this study proves the effectiveness of ferric iron to remove different carbon compounds, suppress methane production in septic environments and remove phosphate. It is postulated that larger scale studies will replicate the findings of our study. We expect to see similar effects of ferric iron on septic wastewater added at the present applied concentration. We will need to refine the type/concentration of  $\text{Fe}^{3+}$  to add to get similar results in situ without causing flow or scaling problems in the tank, or excessive  $\text{Fe}^{3+}$  precipitation in the soil down gradient of the leach field because most of  $\text{Fe}^{+2}$  produced from  $\text{Fe}^{+3}$  reduction will be in solid form, which will precipitate within the tank.

Work will continue to understand the basic mechanism of  $\text{Fe}^{3+}$  reduction in septic wastewater, and to refine the strategy such that it can be retrofitted to existing systems that are not meeting performance goals. Several studies can reveal additional effects of  $\text{Fe}^{3+}$  in onsite wastewater systems such as competition of sulfate reducing and iron reducing bacteria with precipitation competition of FeS and vivianite, combined effects of several types of  $\text{Fe}^{3+}$  on wastewater phosphate and carbon removal, effect of environmentally relevant concentration of  $\text{Fe}^{3+}$  on phosphate removal, methane suppression and carbon oxidation. We believe our study will lead to more challenging and effective research such as recovery of phosphorus from onsite wastewater system as vivianite, removal of pharmaceuticals from onsite wastewater system etc. Phosphate recovery as struvite is well-accepted in traditional wastewater treatment systems. It might be possible to use phosphate recovery as vivianite in onsite wastewater based on the findings of our study. Its effectiveness of removing complex carbon molecules such as lipid, starch, xylose indicates that use of ferric iron amendment to remove complex carbon molecules such as pharmaceuticals can be an effective strategy as the onsite wastewater is a significant source of pharmaceuticals for natural environment.

The research will significantly impact the area of onsite wastewater treatment specifically phosphorus removal. Thus, cost-effective solutions such as ours to onsite wastewater treatment will help to eliminate small scale sources of water pollution. Moreover, this technique can be applied to developing countries where resources, both in terms of money and in terms of infrastructure, are limited. It can impact the sanitation practices and can instigate community level resource recovery both in developed and developing countries. Our proposed research focuses on the development of a “cost-effective” and “integrated” technique of removing several contaminants of interest without any additional footprint requirement.

The role of  $\text{Fe}^{3+}$  reduction in engineered treatment systems is starting to get recognized. Recent publication demonstrated that  $\text{Fe}^{3+}$  reduction is a dominant process for removing odor causing organic acids in swine waste lagoons with  $\text{Fe}^{3+}$  reducer biomass comprising a majority of the organisms present (Coates et al., 2005; Castillo-Gonzalez et al., 2005). Their role in bioremediation has already been well established (Lovley, 2003). It is likely that  $\text{Fe}^{3+}$  reduction has a great deal of utility in engineered wastewater applications; this research is one step towards understanding that utility.

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## APPENDIX

**Table 7:** Name of the clones in iron amended and un-amended samples

HA 1: Uncultured bacterium clone AV5-79 (Uncultured $\beta$ -Proteobacteria)
HA 2: Uncultured Bacteroidetes/Chlorobi group bacterium clone D25_44 (Uncultured Bacteroidetes)
HA 3: Uncultured Acidobacteriaceae bacterium clone D12_13 (Uncultured Firmicutes)
HA 4: Uncultured bacterium clone: MAD-14 (Uncultured $\delta$ -proteobacteria)
HA 5: Uncultured bacterium clone H2-plate 10_G12 (Uncultured Firmicutes)
HA 6: Uncultured bacterium clone E18-966 ( $\alpha$ -Proteobacteria)
HA 7: Uncultured Bacteroides clone 8 (Uncultured Bacteroidetes)
HA 8: Uncultured delta proteobacterium clone EtOH+Fe-c48 (Uncultured $\delta$ -proteobacteria)
HA 9: Uncultured bacterium clone MS044 (Uncultured Firmicutes)
HA 10: Uncultured bacterium clone 5C84 (Uncultured Spirochaetes)
HA 11: Uncultured bacterium clone ncd289d05c1 (Uncultured Firmicutes)
HA 12: Uncultured bacterium clone SBW-29 (Uncultured Firmicutes)
HA 13: Uncultured bacterium clone Tat-08-001_84 (Uncultured $\gamma$ -Proteobacteria)
HA 14: Uncultured bacterium clone R125
HA 15: Uncultured bacterium clone ncd1853h01c1 (Uncultured Actinobacteria)
HA 16: Uncultured bacterium clone 054H04_B_DI_P58 (Uncultured Synergistetes)
HA 17: Uncultured Firmicutes bacterium clone QEDT2CH07 (Uncultured Firmicutes)
HA 18: Uncultured bacterium clone F5K2Q4C04IKMEH (No result)
HA 19: Uncultured bacterium clone Aug05-pVII-C01 (Uncultured $\beta$ -Proteobacteria)
HA 20: Uncultured bacterium clone ncd1900b09c1 (Uncultured Firmicutes)
HA 21: Mycobacterium parascrofulaceum strain GR-4486 (Actinobacteria)
HA 22: Uncultured bacterium clone T2WK15B62 (Uncultured Firmicutes)
HA 23: Uncultured bacterium clone 9V80 16S (Uncultured Firmicutes)
HA 24: Uncultured bacterium clone cs14 (Uncultured $\delta$ -proteobacteria)
HA 25: Uncultured Synergistetes bacterium clone QEDN10AF01 (Uncultured Synergistetes)
HA 26: Uncultured CFB group bacterium clone 44a-B1-42 (Uncultured Bacteroidetes)

**Table 7 (cont.)**

HA 27: Desulfovibrio sp. MS ( $\delta$ -proteobacteria)
HA 28: Uncultured Gammaproteobacteria clone QEDN7DH12 (Uncultured $\gamma$ -Proteobacteria)
HA 29: Uncultured bacterium clone 5S5 (Uncultured $\delta$ -proteobacteria)
HA 30: Uncultured bacterium clone BAZ_aah90e06 (Uncultured Firmicutes)
HA 31: Uncultured bacterium clone FGL12_B89 (Uncultured $\delta$ -proteobacteria)
HA 32: Clostridium difficile BI1 chromosome (Firmicutes)
HA 33: Uncultured bacterium clone T1WK15C46 (Uncultured Firmicutes)
HA 34: Uncultured bacterium clone EEOTU14 (Uncultured Bacteroidetes)
HA 35: Uncultured bacterium clone 5C84 (Uncultured Spirochaetes)
HA 36: Uncultured bacterium isolate DGGE gel band PE_D_21 (Uncultured Firmicutes)
HA 37: Uncultured Desulfobulbaceae bacterium clone HT06Ba03 (Uncultured $\delta$ -proteobacteria)
HA 38: Uncultured bacterium clone 22f02 (Uncultured $\delta$ -proteobacteria/ Acidobacteria)
HA 39: Uncultured bacterium clone E79-1238 (Uncultured $\delta$ -proteobacteria)
HA 40: Alpha proteobacterium pACH81 (Uncultured $\alpha$ -Proteobacteria)
HA 41: Uncultured bacterium clone WBB38 (Uncultured $\alpha$ -Proteobacteria)
HA 42: Uncultured bacterium clone A02 (Uncultured Synergistetes)
HA 43: Uncultured bacterium clone B45 (Uncultured Chloroflexi)
HA 44: Uncultured bacterium clone 22f02 (Uncultured $\delta$ -proteobacteria/Acidobacteria)
HA 45: Uncultured bacterium clone EEOTU7 (Uncultured Firmicutes)
HA 46: Uncultured bacterium clone AR-61 (Uncultured $\beta$ -Proteobacteria)
HA 47: Uncultured Synergistetes bacterium clone QEDN4AF10 (Uncultured Synergistetes)
HA 48: Clostridium perfringens strain: JCM 5239 (Uncultured Firmicutes)
HA 49: Uncultured bacterium clone M40C48 (Uncultured $\beta$ -Proteobacteria)
HA 50: Mycobacterium sp. VC-YC6670 (Uncultured Actinobacteria)
HA 51: Uncultured bacterium clone HH_g04 (Uncultured Firmicutes)
HA 52: Uncultured bacterium clone Aug05-pVII-C01 (Uncultured $\beta$ -Proteobacteria)
HA 53: Uncultured bacterium clone B5_333 (Uncultured Acidobacteria)

**Table 7 (cont.)**

HA 54: Uncultured Clostridiaceae bacterium clone C17 (Uncultured Firmicutes)
HA 55: Thioclava sp. JC56 (Uncultured $\alpha$ -proteobacteria)
HA 56: Uncultured bacterium clone 16saw93-3f06. w2k (Uncultured Firmicutes)
HA 57: Uncultured bacterium clone RT9-ant02 -a12-W (Uncultured Firmicutes)
HA 58: Uncultured bacterium clone RABS_C56 (Uncultured Firmicutes)
HA 59: Bacterium enrichment culture clone BA68 (Uncultured $\beta$ -Proteobacteria)
HA 60: Uncultured bacterium clone SSW_127 (Uncultured Firmicutes)
HA 61: Uncultured Aminomonas sp. clone F10 (Uncultured Synergistetes)
HA 62: Uncultured Firmicutes bacterium clone QEDV1DD11 (Uncultured Firmicutes)
HA 63: Bacterium GC26(2011) (Gammaproteo bacteria)
HA 64: Uncultured Clostridiaceae bacterium clone HrhB91 (Uncultured Firmicutes)
HA 65: Uncultured bacterium isolate d21h4b18 (Uncultured $\delta$ -proteobacteria)
HA 66: Uncultured bacterium clone LC16 (Uncultured Spirochaetes)
HA 67: Uncultured rumen bacterium clone P5_O04 (Uncultured Actinobacteria)
HA 68: Uncultured bacterium clone GW-31 (Uncultured Firmicutes/Proteobacteria)
HA 69: clostridium perfringens strain: JCM 5239 (Uncultured Firmicutes)
HA 70: Uncultured epsilon proteobacterium clone: 1061 (Uncultured Epsilonproteobacteria)
HA 71: Uncultured bacterium clone C70 (Uncultured Firmicutes)
HA 72: Uncultured bacterium clone J5-58 (Uncultured Firmicutes)
HA 73: Uncultured bacterium clone EEOTU17 (Uncultured Synergistetes)
HA 74: Uncultured bacterium clone BS07 (Uncultured Firmicutes)

### **Total Iron Requirement for Phosphorus Removal from OWS:**

The total estimated average daily residential wastewater flow range from 189-265 L/person/day and the typical total phosphorus concentration in the influent wastewater varies from 6-12 mg/L (USEPA, 2002). Eighty percent of total phosphorus is in orthophosphate form (Pouladi, 2011). The molar ratio of  $\text{Fe}^{2+}$ -to- $\text{PO}_4^{3-}$  is 3:2 in accord with the stoichiometry given below through which the  $\text{Fe}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}$  (vivianite) is formed:



Assuming the maximum values of the typical range of flows and phosphorus concentration, the iron requirement can be calculated as:

$$80\% * [\text{Phosphorus Conc. (mg/L)}] / \text{MW(P)} * \text{Molar Ratio} * \text{MW (Fe)}$$

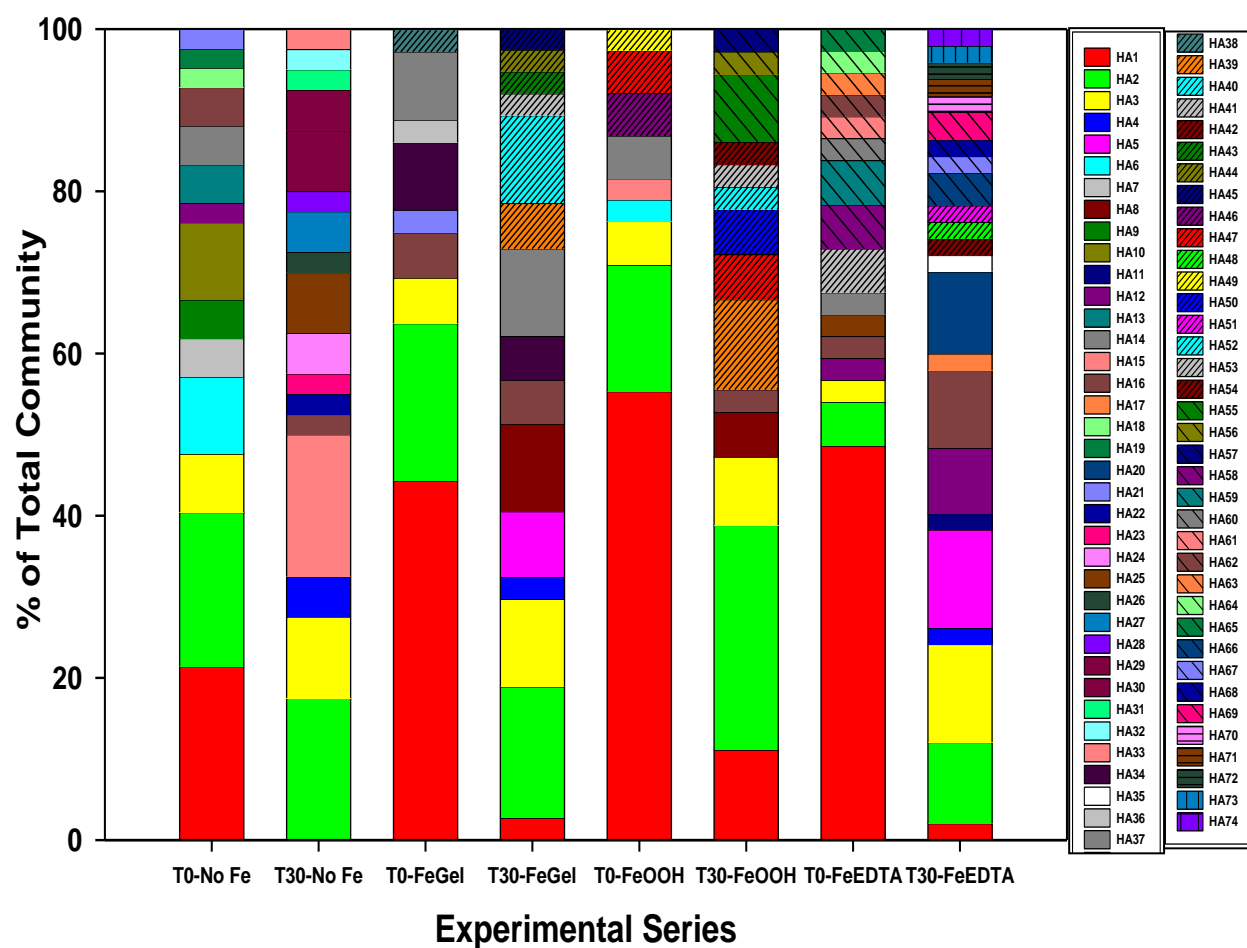
$$= 80\% * (12 * 1/31) * (3/2) * 56 \text{ mg/L} = 26 \text{ mg/L}$$

$$\text{Iron requirement per year} = 26 \text{ mg/L} * 265 \text{ L/person/day} * 2.7 \text{ person} * 365 \text{ days} * (1\text{gm}/1000\text{mg})$$

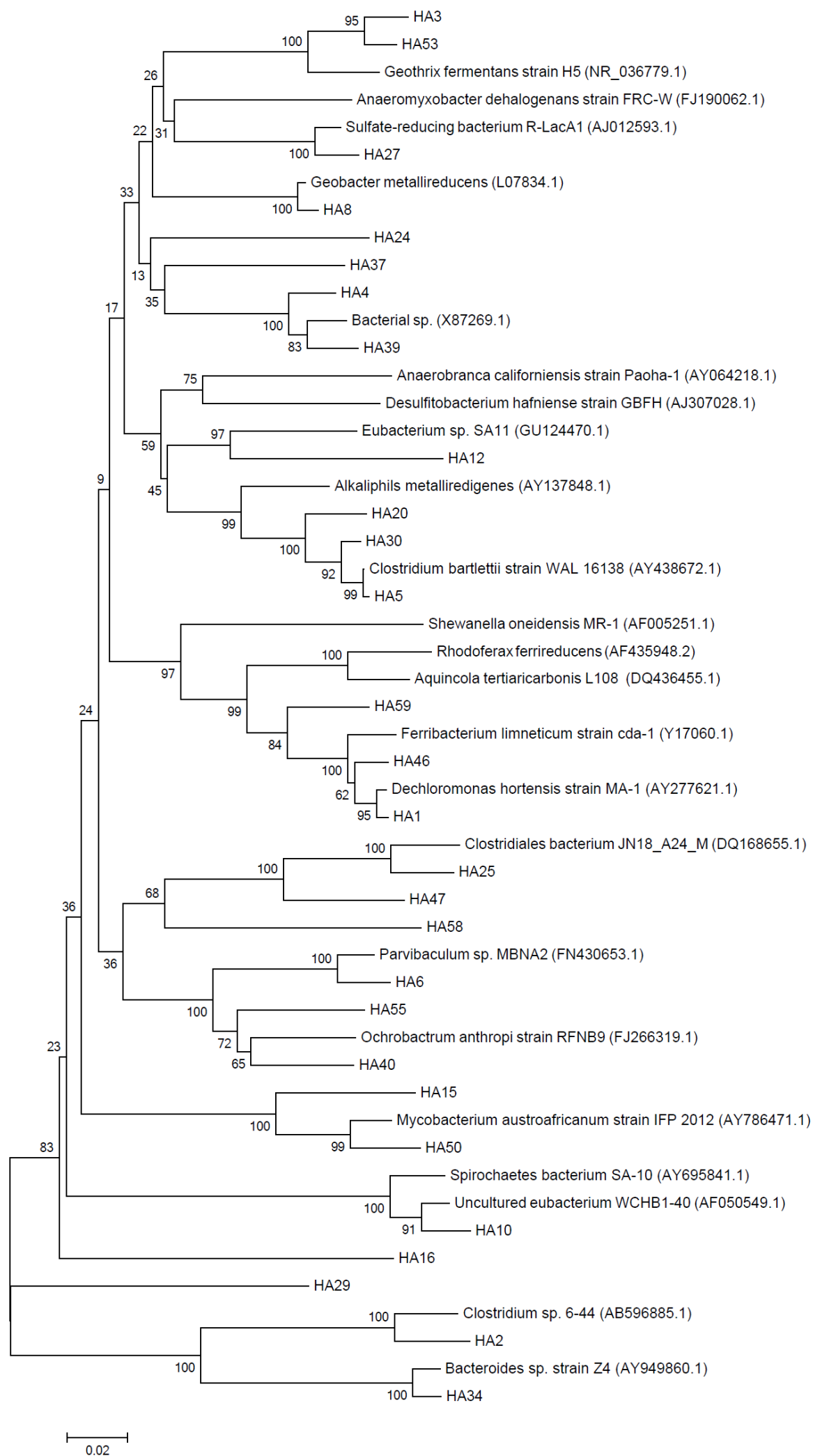
$$= 6790 \text{ gm}$$

$$= 6.79 \text{ Kg}$$

$$\text{Iron requirement per day} = 0.0186 \text{ Kg}$$

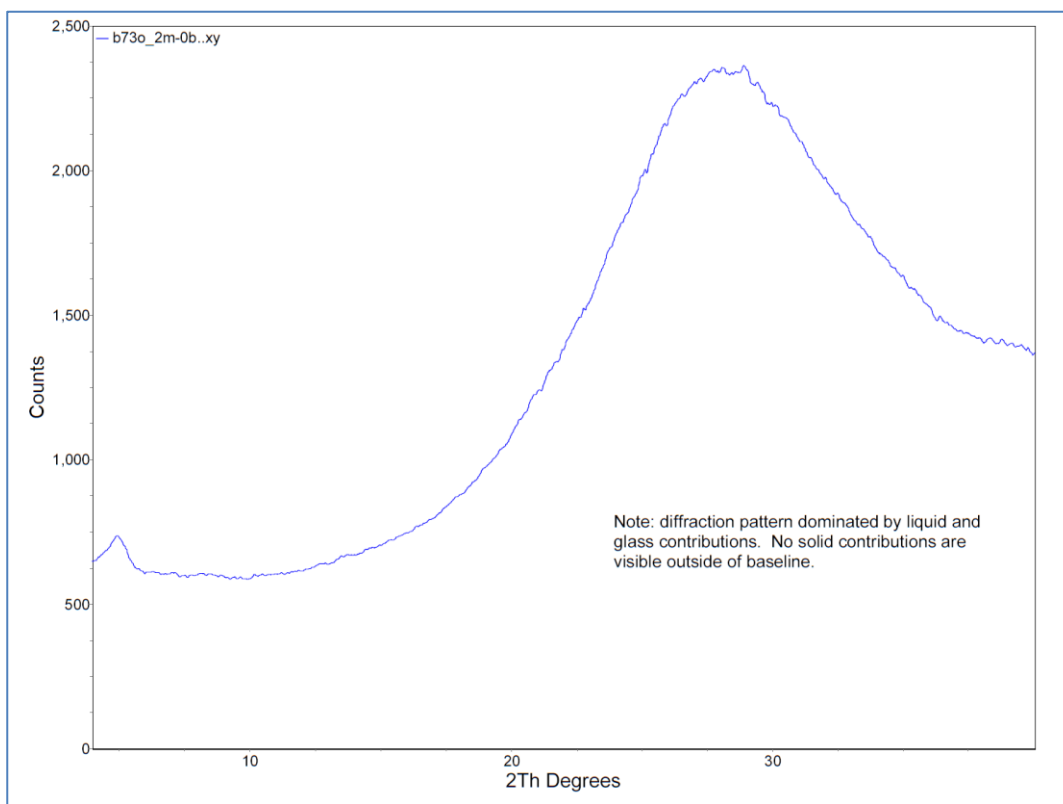


**Figure 30:** Microbial community composition in the iron amended and un-amended incubations based on 16S rRNA gene molecular analysis at the start and end of incubation. The nomenclatures of the microcosms are summarized in Table 7. The closely related phylotype to each clone is shown in parentheses.



**Figure 31:** Phylogenetic relationship of the dominant clones with  $\text{Fe}^{3+}$  reducers, sulfate reducers, and known nitrate reducers. Bootstrap values above 50% (1000 replicates) are presented.

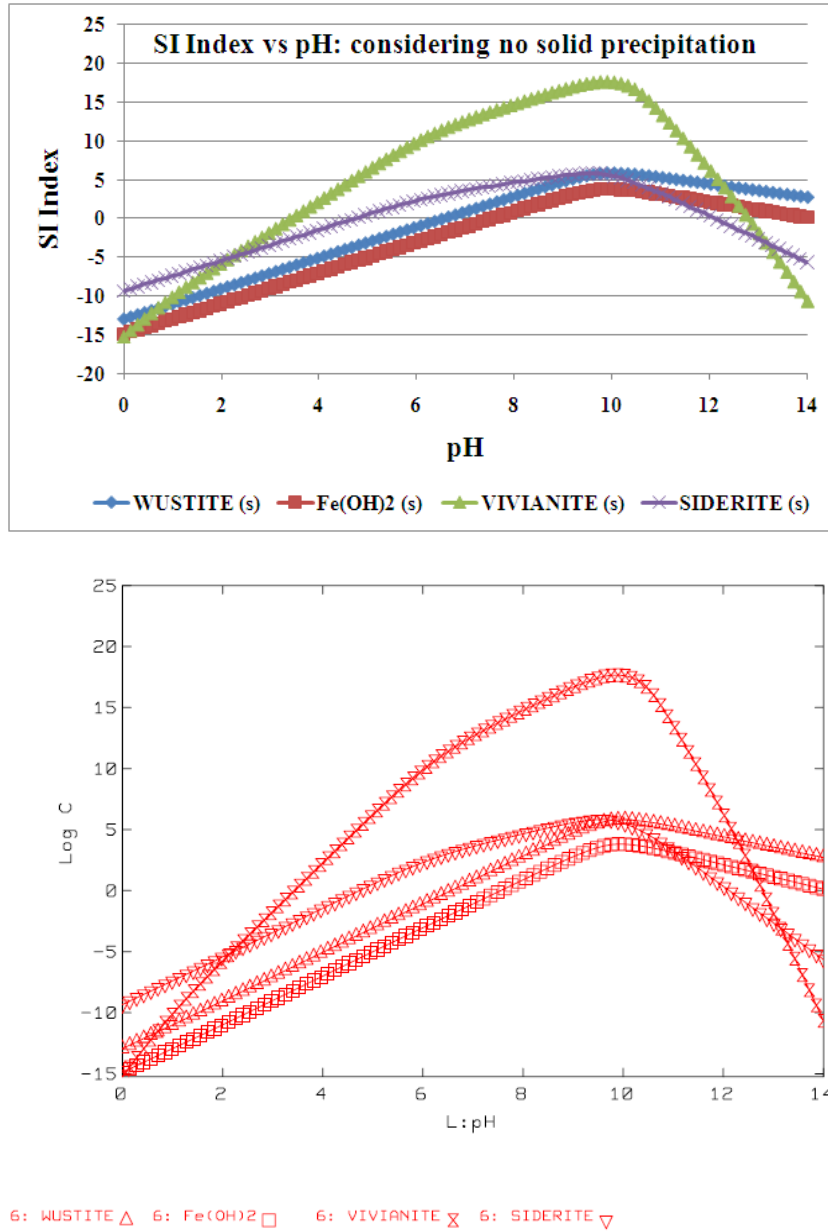




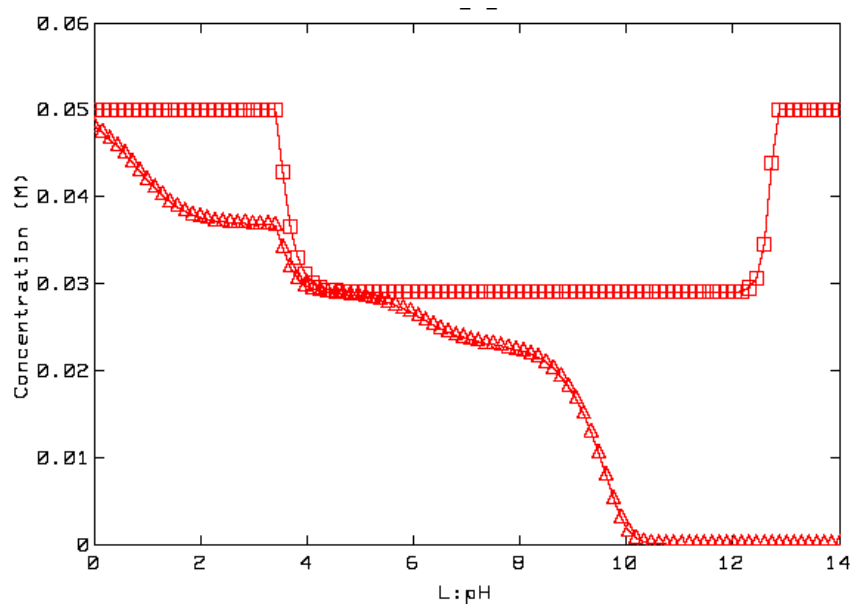
**Figure 32:** Precipitates response from x-ray (blue) of phosphate un-amended sample. Diffraction pattern was dominated by liquid and glass contributions. No mineral contribution was visible and found.

**Table 8:** COD removal due to different  $\text{Fe}^{3+}$  amendment in diluted septic wastewater. Experiment started with 20 mM  $\text{Fe}^{3+}$ . Additional 10 mM  $\text{Fe}^{3+}$  were added at time = 20 days

Amendment	Average COD (mg/L) T = 0 day	Std Dev. (mg/L)	Average COD (mg/L) T = 35 days	Std Dev. (mg/L)	COD Removal (mg/L)
<b>Ferrihydrite</b>	542.2	16.3	325.8	24.5	216.5
<b>Lepidocrocite</b>	554.5	28.5	425.4	34.1	129.1
<b>Fe (III) NTA</b>	547.7	23.7	213.9	24.3	333.8
<b>Fe (III) EDTA</b>	545.4	46.6	371.5	28.9	173.9
<b>Ferrihydrite + AQDS</b>	542.9	66.7	256.6	64.3	286.3
<b>Unamended</b>	549.1	13.2	458.2	18.1	90.9

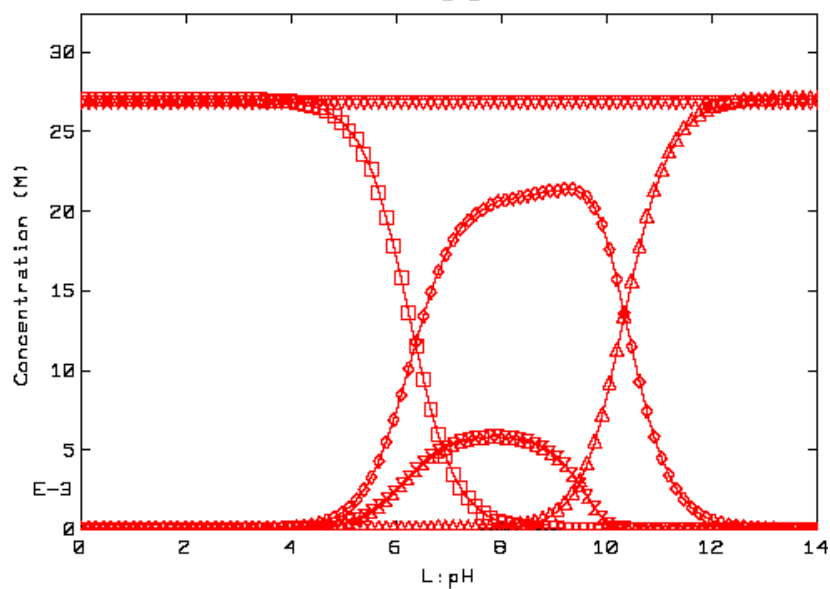


**Figure 33:** Saturation indices in multiple pH values: MINEQL+ modeling (50 mM Fe<sup>2+</sup> and 10 mM PO<sub>4</sub>-P in presence of C<sub>T,CO3</sub> = 27 mM (FeCit media)).



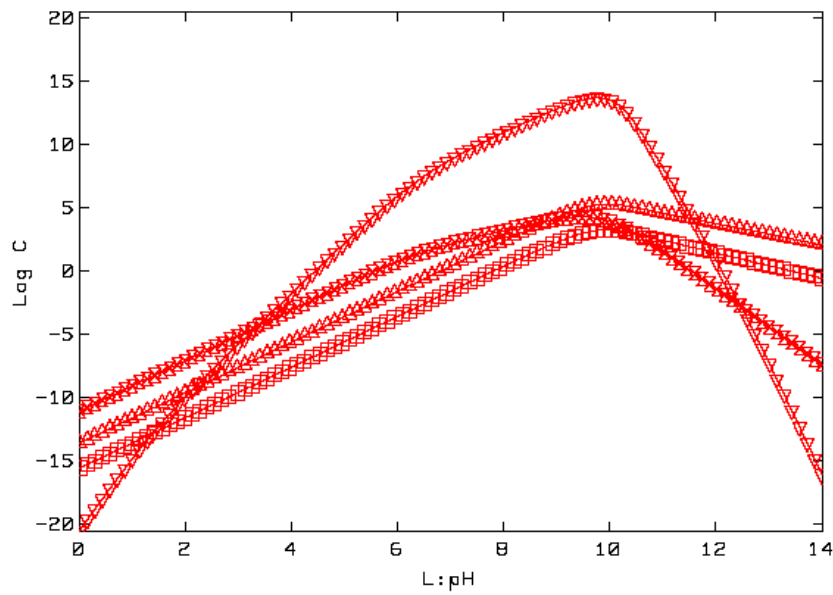
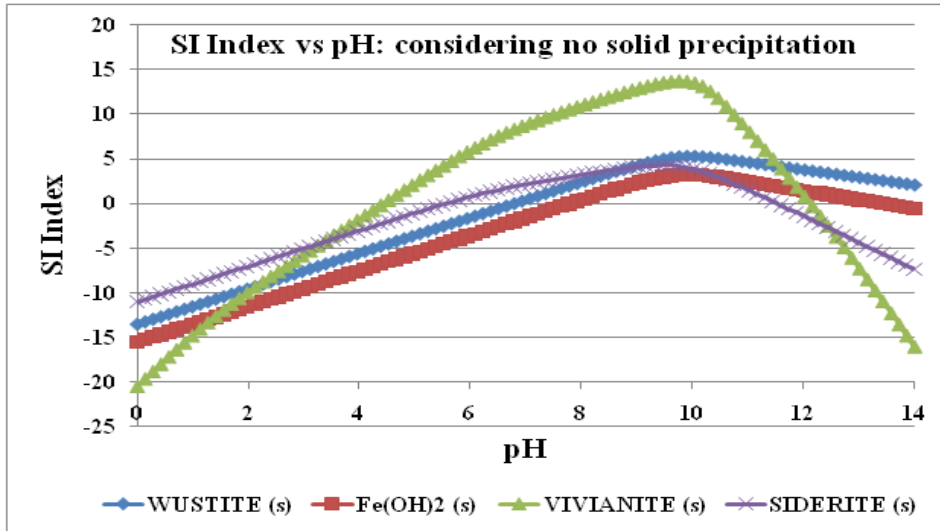
1: Fe(2+) Δ 7: TOTAL Fe (□)

**Figure 34:** TOTFe and  $\text{Fe}^{2+}$  vs pH: MINEQL+ modeling (50 mM  $\text{Fe}^{2+}$  and 10 mM  $\text{PO}_4\text{-P}$  in presence of  $C_{\text{T,CO}_3} = 27$  mM-considering vivianite present).



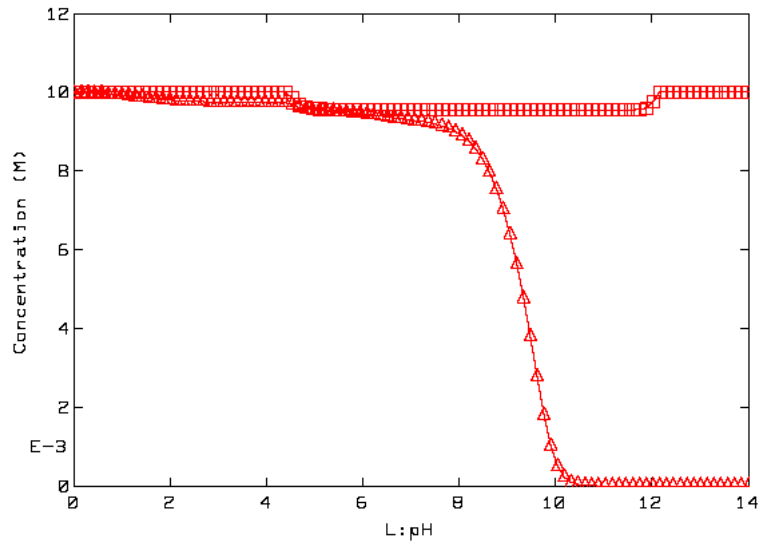
1: CO3(2-) Δ 2: H2CO3 (aq) □ 2: HCO3- ◇ 2: FeHCO3+ × 7: TOTAL CO3 ▽

**Figure 35:**  $\text{CO}_3^{2-}$  speciation diagram: MINEQL+ modeling (50 mM  $\text{Fe}^{2+}$  and 10 mM  $\text{PO}_4\text{-P}$  in presence of  $C_{\text{T,CO}_3} = 27$  mM-considering vivianite present).



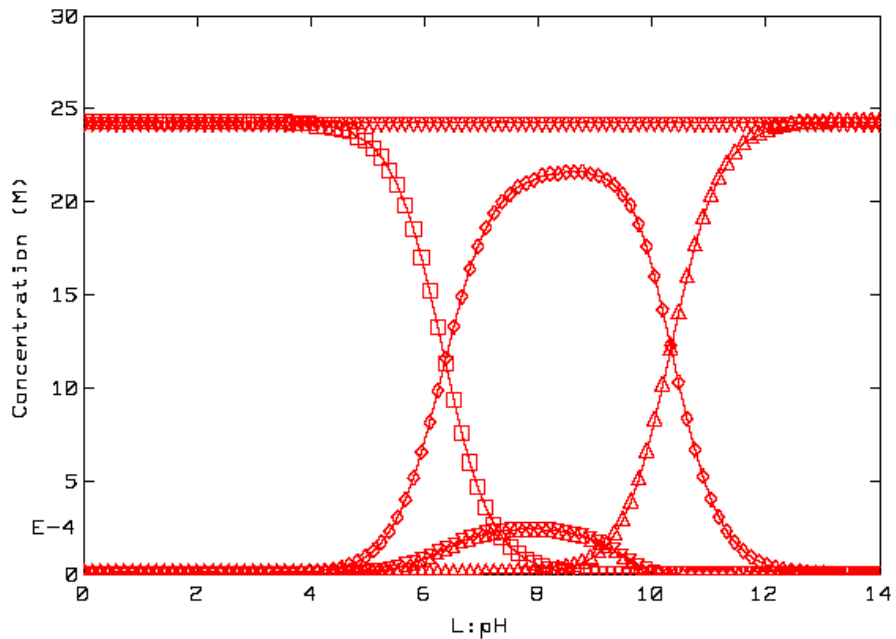
6: WUSTITE  $\Delta$  6: Fe(OH)<sub>2</sub>  $\square$  6: SIDERITE  $\times$  6: VIVIANITE  $\nabla$

**Figure 36:** Saturation indices in multiple pH values: MINEQL+ modeling (10 mM Fe<sup>2+</sup> and 0.306 mM PO<sub>4</sub>-P in presence of C<sub>T</sub>, CO<sub>3</sub> = 2.43 mM septic wastewater).



1: Fe(2+) Δ 7: TOTAL Fe(□)

**Figure 37:** TOTFe and  $\text{Fe}^{2+}$  vs pH: MINEQL+ modeling (10 mM  $\text{Fe}^{2+}$  and 0.306 mM  $\text{PO}_4\text{-P}$  in presence of  $C_{\text{T},\text{CO}_3} = 2.43$  mM- considering vivianite present in septic wastewater).



1: CO3(2-) Δ 2: H2CO3 (aq) □ 2: HCO3- ◇ 2: FeHCO3+ × 7: TOTAL CO3 ▽

**Figure 38:**  $\text{CO}_3$  speciation diagram: MINEQL+ modeling (10 mM  $\text{Fe}^{2+}$  and 0.306 mM  $\text{PO}_4\text{-P}$  in presence of  $C_{\text{T},\text{CO}_3} = 2.43$  mM considering vivianite present in septic wastewater).